Development and evaluation of tools to analyze the response of tomato (*Solanum lycopersicum* L.) rootstocks under nitrogen deficiency in a breeding context

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"Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails.

Explore. Dream. Discover".

Mark Twain.

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Contents

Acknowle	dgements	i
Contents		iii
Abbreviat	ions	vii
List of figu	Jres	xi
List of tab	les	xiii
Preface		1
General i	ntroduction	5
1.1.	Habitat, diversity and breeding	5
1.2.	Some considerations on horticulture process	9
1.2.1	L. Grafting	9
1.2.2	2. Aeroponic growing method	12
1.3.	Chlorophyll fluorescence for assessment of nitrogen status	13
1.3.1	L. Evaluation of crop nitrogen status	15
1.4.	The vegetative development	20
1.4.2	L. Shoot architecture of tomato	21
1.4.2	2. Root growth, development and architecture	22
1.5.	Mineral nutrition of tomato	24
1.5.1	 Nitrogen in plants ecosystem 	25
1.5.2	2. Nitrogen nutrient uptake systems	28
1.6.	Root architecture responses to nitrogen nutrition	32
Objective	s and outline	35
2.1.	Rationale of the thesis	35
2.2.	Outline of the thesis	36
Determin	ation by near infrared microscopy of the nitrogen and carbon o	ontent of
tomato (S	Solanum lycopersicum L.) leaf powder	39

3.1.	Introduction	41
3.2.	Material and Methods	43
3.2.	.1. Plant material and growth conditions	43
3.2.	.2. NIR microscopy	45
3.2.	.3. Reference analysis	46
3.2.	.4. Statistical analysis	46
3.3.	Results and discussion	49
3.3.	.1. Spectra description	49
3.3.	.2. Reference analysis	52
3.3.	.3. NIRM calibration and validation	53
3.4.	Conclusions	59
Assessme	ent of diagnostic optical tools for the evaluation of N content in to	mato
(Solanum	n lycopersicum L.) cuttings	63
4.1.	Introduction	65
4.2.	Materials and methods	68
4.2.	.1. Plant material and growth conditions	68
4.2.	.2. Biomass and leaf area measurements	69
4.2.	.3. Optical sensor measurements	70
4.2.	.4. NIR microscopy approach	72
4.2.	.5. Statistical analysis	73
4.3.	Results and discussion	73
4.3.	.1. Plant growth	73
4.3.	.2. Photosynthetic pigments concentration, chlorophyll index a	nd
nitro	ogen content	76
4.3.	.3. Fluorimeter	79
4.3.	.4. Multiplex	80
4.3.	.5. Principal component analysis	84
4.3.	.6. Prediction of N content	

iv

C-	-	4	_	-	4.0
CO	n	υ	e	n	ιs

4.3.7	Conclusion	
A genetic	analysis of rootsto	ck contribution to biomass production of tomato
(Solanum	<i>lycopersicum</i> L.) ur	nder nitrogen deficiency91
5.1.	Introduction	93
5.2.	Materials and met	hods96
5.2.1	Plant materia	al and grafting96
5.2.2	2. Plant growth	conditions in greenhouse97
5.2.3	8. Plant biomas	s measurements98
5.2.4	. Optical meas	urements98
5.2.5	. Root structur	re architecture parameters evaluation
5.2.6	5. Statistical an	alysis100
5.2.7	. QTL analysis	
5.3.	Results and discus	sion101
5.3.1	Phenotyping	data
5.3.2	. Heritability	
5.3.3	B. QTL analysis	
5.4.	Conclusion	
Model-ba	sed conception of i	root traits of tomato (Solanum lycopersicum L.)
rootstock	S	
6.1.	Introduction	
6.2.	Materials and met	hods
6.2.1 eval	Plant materia Jation 117	al and root structure architecture parameters in situ
6.2.2	. Root growth	modelling120
6.3.	Results and discus	sion122
6.3.1	Plant phenot	ypes122
6.3.2	. Plant modeli	ng workflow124

V

	6.3.3.	Results of the parameterization of the whole rootstock p 129	opulation
6.4	. Conc	clusion	131
Concl	usions and	d perspectives	133
Biblio	graphy		139
List of	f publicati	ions	179
Currio	ulum vita	ie	

vi

Abbreviations

AAP	Amino acid permease
AMT	Ammonium transporter
ANR	Nitrate regulated
ANTH	Anthocyanins
В	Blue
BGF	Blue-Green fluorescence (also YF)
Ca	Concentration in chlorophyll a
Cb	Concentration in chlorophyll b
Ccarot	Concentration in carotenoids
CCM	Chlorophyll index
CV	Cross-validation
Df	Degree of freedom
DW	Dry weight
EC	Electrical conductivity
Exp	Exponential
FER	Fluorescence excitation ratio
FERARI	Anthocyanins relative index
FLAV	Flavanols
Fm	Maximun fluorescence
FO	Initial fluorescence

vii

FR	Fresh weight
FRF	Far-Red fluorescence
FV	Variable fluorescence
G	Green
H ²	Heritability
HATS	High affinity transport system
HWidth	Horizontal width
IM	Interval mapping
InterLR	Distance between successive lateral roots
IVIA	Instituto valenciano de investigaciones agrarias
LA	Leaf area
LAR	Leaf area ratio
LATS	Low affinity transport system
LHT	Lysine-histidine transporter
LOD	logarithm of odds
Max	Maximum
Mean	Average
MEP	Methylamine permease
Min	Minimum
MIR	Mid infrared
MQM	Multiple QTL Mapping
MRL	Maximum root length
Ν	Nitrogen
NASA	National aeronautics space administration
Nb	number

Abbreviations

NBI	Nitrogen balance index
NPQ	Non-photochemical quenching
NIR	Near infrared
NIRM	infrared microscopy
NIRS	Near infrared spectroscopy
NRT	Nitrate Transporter
PAR	Photosynthetically active radiation
PCA	Principal components analysis
PLS	Partial least squares
QTL	Quantitative trait loci
R	Red
R ²	Determination coefficients
R ² c	Determination coefficients of calibration
R ² _{cv}	Determination coefficients of cross-validation
R ² p	Determination coefficients of prediction
RDW	Root dry weight
RF	Red fluorescence
RFW	Root fresh weight
RH	Relative humidity
Rh	Rhesus
RMSE _p	Root mean square of prediction errors
RootDens	Root density
RootNb	Adventitious root number
RootAngle	Adventitious root angle
RPD	Ratio of prediction to deviation

Abbreviations

RPDc	Ratio of prediction to deviation of calibration
RPD _{cv}	Ratio of prediction to deviation of cross-validation
R_S	Root:shoot
RSA	Root system architecture
RSD	Residual standard deviation
RWC	Root water content
SD	Standard deviation
SDW	Shoot dry weight
SEC	Standard error of calibration
SEC _{CV}	Standard error of cross-validation
SEL	Standard error of the reference method
SEP	Standard error of prediction
SFR	Simple fluorescence ratio
SFW	Shoot fresh weight
SLA	Specific leaf area
SWC	Shoot water content
UV	Ultra-violet
WL	Wavelengths
YF	Yellow fluorescence (also BGF)

Х

List of figures

Figure 1-1. Schematic representation of standing aerated hydroponic growing (A)
and aeroponic growing methods (B)13
Figure 1-2. Energy budget of a typical leaf14
Figure 1-3. Variable chlorophyll fluorescence parameters
Figure 1-4. Schematic presentation of key enzymes involved in nitrogen managem-
ent in (A) young and (B) senescing leaves
Figure 2-1: Schematic outline of the thesis
Figure 3-1. Workflow of the NIRM analysis performed45
Figure 3-2. Comparison between the near-infrared spectra of one of our samples
analyzed by NIRM instrument and by NIRS classical instrument
Figure 3-3. Plot of % N in the Solanum lycopersicum L. samples analyzed in the
calibration (A) and in the validation (B) stages55
Figure 4-1. Number of leaves, plant dry weight, shoot dry weight and root dry weight
as a function of thermal units74
Figure 4-2. Effect of the nitrogen input on the concentration of chlorophyll a,
chlorophyll b and carotenoids measured at harvest. Temporal evolution of the
chlorophyll index by CCM-200 and of the predicted nitrogen value by NIRM for the
six nitrogen inputs

xi

Figure 4-3. Effect of the nitrogen input (mM) on the initial fluorescence (Fo), variable
fluorescence (Fv), maximum fluorescence (Fm) and variable/maximum fluorescence
(Fm/Fv) ratio
Figure 4-4. Temporal evolution of the eight Multiplex variables selected for further
analysis
Figure 4-5. Scores of the eight variables in the first two principal components and
trajectories of the different nitrogen dose in the PC1-PC2 plane
Figure 4-6. Graphical representation of a principal component analysis performed
on the complete set of variables analyzed
Figure 5-1. The morphological diversity of root systems in the aeroponic
platform
Figure 5-2. Distribution of genotypic means in low and high N conditions (I) 105
Figure 5-3. Distribution of genotypic means in low and high N conditions (II) 106
Figure 6-1. Illustration of the morphological diversity observed in aeroponics within
the tomato population

List of tables

Table 3-1. The principal chemical compounds used in nitrogen solutions
Table 3-2. Characteristics of the calibration and validation sets 52
Table 3-3. Calibration models differences
Table 3-4. Characteristic of the NIRM models constructed
Table 3-5. Statistics of the NIRM model 3 validation for the nitrogen determination 56
Table 3-6. Characteristics and statistics of the final NIRM model
Table 4-1. The principal chemical compounds used in nitrogen solutions 69
Table 4-2. Characteristic of the physical and the physiology (grey background)
parameters directly related to the physiological status of plants provided by the
Multiplex71
Table 4-3. Variance analyses of growth-related variables 75
Table 4-4. Variance analyses of photosynthetic pigments concentration, chlorophyll
index and NIRM prediction
Tableau 4-5. Variance analysis of fluorescence variables 80
Table 5-1. List of traits evaluated under optimal and low N and trait code for QTL
analysis in the experiment by UCL103
Table 5-2: Evaluation of interaction between the repetition of the experiment and
treatment

xiii

Table 5-3. Broad sense heritabilities, computed separately for each N supply (low
and high N), at the experiment-wise level (Global) and for the response to N supply
(Response) 107
Table 5-4. List of QTLs detected by interval mapping (5% overall significance
level)
Table 6-1. Root parameters of the stem, adventitious root, lateral root and transition
stem for growth, branching, growth direction and transition phase in RootTyp
software

xiv

Preface

With a world production of more than 164 million tons, tomato is one of the most consumed vegetables in the world (FAOSTAT, 2017). It is the seventh most important crop in the world after maize, rice, wheat, potatoes, soybeans and cassava. The area dedicated to tomato production culture has doubled during the last 20 years. In the past, Europe and the Americas represented the most important producers. Nowadays, China and India dominates the world tomato market, followed by USA, Turkey, Egypt, Iran, Italy, Brazil, Spain and Mexico (Bergougnoux, 2014; FAOSTAT, 2017). The highest yield are achieved in northern Europe countries such as Belgium, Netherlands, Ireland, United Kingdom, Iceland (FAOSTAT, 2017), where tomatoes are produced under controlled greenhouse conditions.

Since the beginning of the century, the worldwide tomato production has increased to cope with a global average consumption of 20.3 kg/capita/year in 2011 (FAOSTAT, 2017). The largest consumption are observed in Turkey and Egypt, where it exceeds 91 kg/capita/year (FAOSTAT, 2017).

The tomato is an herbaceous plants species of the Solanaceae family, native to northwestern South America. This family includes more than 3000 species as potatoes, eggplants, petunias, tobacco, peppers (*Capsicum*) and Physalis which are of economic interest. The Solanum genus, which comprises 1250 to 1700 species distributed on tropical continents and on all temperate regions, is the largest genus in the Solanaceae family (Bergougnoux, 2014, Knapp and Peralta, 2016). Species of the Solanum genus display an exceptional morphological and ecological diversity.

1

They include crop species as well as species producing poisonous or medicinal compounds (Weese and Bohs, 2007; Bergougnoux, 2014; Gebhardt, 2016).

The popularity of the tomato as a research model organism has increased over the years, due to several characteristics (Davis *et al.*, 2008a; Lozano *et al.*, 2009; Pineda *et al.*, 2010; The Tomato Genome Consortium, 2012; Bergougnoux, 2014; Ichihashi and Sinha, 2014; Périlleux *et al.*, 2014; Gebhardt, 2016; Knapp and Peralta, 2016):

- its relatively short life cycle,
- the ease of manual pollination and hybridization,
- its photoperiod insensitivity,
- its adaptability to a wide range of conditions, which is extremely valuable for the study of various abiotic stresses (cold, drought,...),
- its high self-fertility and homozygosity,
- its ability to be propagated asexually by grafting,
- the possibility to regenerate whole plants from different parts of the plant,
- its relatively small genome with a lack of gene duplication,
- the availability of its genome sequence,
- its agronomic value.

Tomato is a nice complement to the main model species, *viz*. Arabidopsis, maize and rice, which it is phylogenetically distant, because it has some specific morphological traits, flowering and fructification processes, it is capable of indeterminate growth due to a reiterative switches from vegetative to reproductive phases, it can be grafted very easily and large collections of mutants are available (Quinet *et al.*, 2006; Carrari *et al.*, 2007; Lozano *et al.*, 2009; Ichihashi and Sinha, 2014; Gebhardt, 2016; Knapp and Peralta, 2016).

Many biotic and abiotic stresses affect the physiology, growth and development of tomato and cause significant yield losses (Kraiser *et al.*, 2011). In particular, an

2

Preface

adequate supply of nutrients under naturally fluctuating environmental conditions is one of the most significant challenges for crop management. Grafting has been adopted widely and early by tomato growers, due to the identification of rootstocks which provide stability and tolerance against chilling, suboptimal and supraoptimal temperature (Rivero *et al.*, 2003a; Rivero *et al.*, 2003b; Schwarz *et al.*, 2010; Ntatsi *et al.*, 2013; Ntatsi and Savvas, 2014), salt stress (Rivero *et al.*, 2003b; Estan *et al.*, 2004; Martinez-Rodriguez *et al.* 2008; Albacete *et al.*, 2019; Asins *et al.*, 2010; Colla *et al.*, 2010c; Keatinge *et al.*, 2014; Albacete *et al.*, 2015a), nutrient stress (Rivero *et al.*, 2003b; Davis *et al.*, 2008a; Savvas *et al*, 2011; Schwarz *et al.*, 2013; Albacete *et al.*, 2015b; Al-Harbi *et al.*, 2016), drought (Keatinge *et al.*, 2014; Al-Harbi *et al.*, 2016; Yao *et al.*, 2016) and soil borne diseases (Rivero *et al.*, 2003b; Louws *et al.*, 2010; Foolad and Panthee, 2012; Borgognone *et al.*, 2013; Keatinge *et al.*, 2014).

Nitrogen is one of the main mineral constituent of plants and its availability is a major factor limiting plant growth. Soil nitrogen is characterized by (1) a biochemical cycle involving pools of different chemical forms which are not all equally available for the plant and (2) the high mobility in soil of the nitrogen that is largely taken up by crops (Kraiser *et al.,* 2011). The consequences of a mismatch between the spatial and temporal distributions of nitrogen and roots are both reduced availability for the plant and an increased risk of leaching to groundwater.

Breeding strategies for improved abiotic stress tolerance and water and nitrogen uptake are focusing on the production of new rootstock and a better understanding of environmental constraints (King *et al.*, 2010). For example, improved uptake of nutrients and tolerance to salt, organic pollutants, flooding and thermal stress have been achieved by the selection of appropriate rootstock and scion combinations (Martinez-Rodriguez *et al.*, 2008; King *et al.*, 2010; Schwarz *et al.*, 2010; Borgognone *et al.*, 2013; Schwarz *et al.*, 2013; RootOPower, 2017). The present thesis was conducted in the framework of the EU-FP7 project RootOPower (2012 - 2015) which aimed at developing a multidisciplinary suite of innovative tools targeting root system traits to enhance agronomic stability and sustainability of tomato under multiple and combined abiotic stresses: salinity, water stress, soil compaction and low fertilizer (N, P, K) input. The specific objective of this thesis was to evaluate the genetic determinism of growth and root architecture in response to nitrogen deficiency. The scientific strategy was based on a population of 144 different rootstocks: six accessions from S. lycopersicum ('Cerasiforme') and S. pimpinellifolium, selected for drought tolerance (sourced from The World Vegetable Center, AVRDC); nine introgression lines from S. lycopersicum × S. pennellii and × S. habrochaites, selected for high root/shoot ratio, salinity and drought tolerances (sourced from The Tomato Genetics Resource Center, TGRC); a population of 129 recombinant inbred lines (RILs) derived from a salt sensitive genotype of S. lycopersicum var. cerasiforme and a salt tolerant line from S. pimpinellifolium L. (Monforte et al., 1997), recently developed by Instituto Valenciano de Investigaciones Agrarias (IVIA), which were either self-grafted or grafted with a commercial tomato cultivar (Solanum lycopersicum L. 'Boludo F1', 'Monsanto') as common scion.

This work is presented into three main parts. The first part introduces the subject, gives the objectives and the outline of the thesis. The second part displays the results of the thesis in four chapters: i) the determination by near infrared microscopy of the nitrogen and carbon content in leaf powder, ii) the assessment of diagnostic optical tools for the evaluation of N content, iii) a genetic analysis of rootstock contribution to biomass production and iv) the model-based conception of root traits. Finally, the third part of this thesis discusses the obtained results and formulates the conclusion and future perspectives.

4

Chapter 1

General introduction

Tomato is an annual or short-lived perennial herbaceous plants species native from northwestern South America and widely cultivated for its fruit. It belongs to the Solanaceae family which includes more than 3000 species with a large diversity in development, organ morphology, metabolism and geographic distribution. The Solanum genus, to which cultivated tomato belongs, is the largest genus of the Solanaceae (1250 to 1700 species) (Bergougnoux, 2014). Many of these species have high economic, nutritional and agricultural importance (*e.g.* potato, eggplant).

1.1. Habitat, diversity and breeding

Wild tomato species are native of the coast and Andean Highlands of western South America, corresponding to central Ecuador, Peru, northern Chile and the Galapagos Islands (Bergougnoux, 2014; Knapp and Peralta, 2016). They grow in a variety of habitats, as indicated by their geographical distribution ranging from sea level on the Pacific coast up to 3300 m above sea level in the Andean Highlands, and from arid to rainy climates (Bergougnoux, 2014; Knapp and Peralta, 2016). Tomatoes have been initially classified in two species: *Solanum pimpinellifolium* and *Solanum Lycopersicum* (Bailey, 1949; Knapp and Peralta, 2016). S. *Lycopersicum* is the main tomato species commercialized (Madhavi and Salumkhe, 1998). Later, Taylor (1986) recognized several additional species of tomato such as S. *cheesemaniae*, S.

5

galapagense, S. chilense, S. chmielewskii, S. habrochaites, S. pennellii, S. neorickii, S. arcanum, S. corneliomuelleri, S. huaylasense and S. peruvianum (Peralta and Spooner, 2005; Spooner *et al.*, 2005; Gebhardt, 2016; Knapp and Peralta, 2016). Tomato wild species are adapted to particular climatic and soil types typical of narrow and isolated valleys which are their natural area. The Andean geography and the diverse climates and ecological habitats contributed together to wild tomato diversity (Bergougnoux, 2014; Knapp and Peralta, 2016). The recent study of Nakazato and Housworth (2011) based on the two closely related wild tomato species S. *lycopersicum* and S. *pimpinellifolium* seems to confirm this hypothesis. This diversity is manifested through morphological, physiological and sexual characteristics (Peralta and Spooner, 2005; Spooner *et al.*, 2005; Knapp and Peralta, 2016).

Species diversification and conservation is largely influenced by the mating system. In wild tomatoes, there is a strong correlation between self-incompatibility and the degree of outcrossing, allelic diversity, floral display and degree of stigma exsertion (Peralta *et al.*, 2008; Knapp and Peralta, 2016). An exserted stigma above the anthers promotes outcrossing by buzz pollination, whereas a recessed stigma below the anthers promotes self-fertilization (Chen and Tanksley, 2004; Knapp and Peralta, 2016). The mating system of tomato evolved from self-incompatible, as the ancestral condition, to self-compatible (Jones, 2008; Knapp and Peralta, 2016). Selfincompatible populations display higher degree of diversity, larger flower parts and exserted stigma, whereas self-compatible populations have a reduced genetic diversity, smaller flower parts and little or no stigma exsertion (Knapp and Peralta, 2016).

The domestication of wild species started about 10.000 years ago (Doebley *et al.*, 2006), and marked the onset of a phase of diversity loss. 4.000 years ago, our ancestors had completed the domestication of major crop species, including rice,

General introduction

wheat, and maize (Doebley *et al.*, 2006). The domestication syndrome varies depending on culture considered but generally includes compact growth habit, increased earliness, reduction/loss of seed dispersal and dormancy, changes in photoperiod sensitivity, synchronized flowering, a decrease in bitter substances in edible structures, gigantism and increased morphological diversity in the consumed part of the crop (Doebley *et al.*, 2006; Bergougnoux, 2014; Lin *et al.*, 2014). Nowadays, modern agriculture has extended this process through a drastic selection for a wide range of morphological and physiological traits. Population bottlenecks that were common events during domestication have reduced diversity in the cultivated germplasm and continued selection has maintained this trend for loss of diversity (Bauchet and Causse, 2012; Lin *et al.*, 2014; Doebley *et al.*, 2006; Knapp and Peralta, 2016).

Tomato breeding aims at producing new cultivars that are adapted to particular conditions, such as soilless culture, high tropical temperatures and field or greenhouse conditions. In particular, cultivars have been developed for the fresh tomato and for the processed tomato markets. Tomatoes for the processed market are mainly cultivated in fields, whereas fresh tomatoes are grown either in fields or in greenhouses with or without temperature control. Breeding objectives have evolved over time following the modifications of culture systems, yet the three main objectives remain, *viz*. the adaptability to the environment, the resistance to pests and diseases and the fruit yield and quality (Lee *et al.*, 2010; Bergougnoux, 2014; Keatinge *et al.*, 2014). The breeding history of tomato has gone through four main phases: breeding for yield in the 1970's, for shelf-life in the 1980's, for taste in the 1990's and since then for nutritional value (Bauchet and Causse, 2012; Bergougnoux, 2014).

Over the past 50 years of selection, the tomato plant and its fruit characteristics have substantially changed. Varieties available today for use by the food-processing industry are characterized by a wide range of features to achieve commercial value, nutritional quality and resistance to biotic and abiotic stresses. Conventional breeding is largely based on the exploitation of heritable variation. However, understanding the mechanisms supporting an interesting agronomic trait has also proven useful to build innovative breeding trajectories. Typically, when these mechanisms are complex and multigenic, quantitative trait loci (QTL) analysis allows to dissect the genetic variability according to the genotype at individual marker loci and to identify candidate genes (Collard et al., 2005; Szczechura et al, 2011; Bauchet and Causse, 2012; Foolad and Panthee, 2012; Shirasawa and Hirakawa, 2013; Lin et al., 2014; Gebhardt, 2016). In particular, understanding the molecular basis of the various traits of the domestication syndrome has been influential on several breeding programs for fruit shape, color, acidity, shelf life, resistance to bruising and high lycopene content (Bauchet and Causse, 2012; Foolad and Panthee, 2012; Shirasawa and Hirakawa, 2013; Lin et al., 2014; Gebhardt, 2016).

Recently, allele mining and the screening of old varieties and wild relatives for alleles of agronomic importance have become more common strategies to recover valuable alleles that were excluded during domestication and selection (Kumar *et al.*, 2010). They have led to the identification of major genes and quantitative trait loci involved in biotic and abiotic stress tolerance. With the expansion of organic food, there is also a growing interest for the use of heirloom varieties (old standard garden varieties).

1.2. Some considerations on horticulture process

In field conditions, tomato is commonly grown in open fields or with various plastic culture systems. In greenhouse, tomato was initially grown in soils, yet hydroponic growing systems are now largely used (Jones, 2008; Wyenandt *et al.*, 2016). Whether in fields or in greenhouses, tomato can be grown successfully in a wide range of conditions. It grows in many substrates such as soils, modified soils, soilless mixes or organic substrates and, in hydroponic systems, in bags or buckets of sand, gravel, perlite, pine bark, or rockwool slabs (Jones, 2008; Wyenandt *et al.*, 2016). Many factors affect tomato yield. Abiotic factors comprise the intensity and spectral characteristics of light, the photoperiod, the air and root temperatures, the relative atmospheric humidity and the nutrient element availability over the life of the tomato plant. Biotic factors include disease and insect infestation. In the horticulture production context, the cultivar and grower skill are also important factors of the tomato crop performance.

1.2.1. Grafting

Grafting has been early adopted in tomato production. Through the fusion of a shoot (scion) and a root system (rootstock) that can have different genotypes, the technique allows to circumvent a major constraint in genetics, *viz*. to combine aerial productivity with root rusticity and tolerance traits in the same genotype. Recourse to grafting has allowed breeders to select elite scions independently from rootstocks which have been chosen based on their root-related characteristics.

One of the most important application of grafting is the achievement of tolerance to soil-borne diseases. Rootstocks have been selected for their excellent tolerance to

Fusarium, Verticillium, Phytophthora, Pseudomonas, Didymella bryoniae, Monosporascus cannonballus and nematodes (Edelstein et al., 1999; Cohen et al., 2000; Ioannou, 2001; Trionfetti Nisini et al., 2002; Blestos et al., 2003; Rivero et al., 2003b; Cohen et al., 2005; Morra and Bilotto, 2006; Cohen et al., 2007; Crinò et al., 2007; Louws et al., 2010; Szczechura et al., 2011; Foolad and Panthee, 2012; Borgognone et al., 2013; Keatinge et al., 2014).

Another application has been the promotion of plant vigor, using large and vigourous rootstocks which enhance water and nutrients absorption (Lee and Oda, 2003; Davis *et al.*, 2008a; Salehi-Mohammadi *et al.*, 2009; Savvas *et al.*, 2010; Al-Harbi *et al.*, 2016). In tomato, fresh fruit weight increases of 50 - 55% have been reported in grafted plants as compared to own-rooted plants (Chung and Lee, 2007). These yield increase were substantially correlated with the maintenance of plant vigor until late in the growing season (Lee *et al.*, 2010, Al-Harbi *et al.*, 2016).

Another important target for vegetable growers is the tolerance to various abiotic stresses. Rootstocks have been used to induce resistance against low and high temperatures (Rivero *et al.*, 2003a, Rivero *et al.*, 2003b; Venema *et al.*, 2008; Schwarz *et al.*, 2010; Ntatsi *et al.*, 2013; Ntatsi and Savvas, 2014), enhance nutrient uptake (Rivero *et al.*, 2003b; Davis *et al.*, 2008a; Colla *et al.*, 2010a; Savvas *et al.*, 2010; Schwarz *et al.*, 2013; Al-Harbi *et al.*, 2016), increase synthesis of endogenous hormones (Dong *et al.*, 2008), improve water use efficiency (Rouphael *et al.*, 2008a; Schwarz *et al.*, 2010; Al-Harbi *et al.*, 2016), reduce uptake of persistent organic pollutants from agricultural soils (Otani and Seike, 2006; Otani and Seike, 2007; Schwarz *et al.*, 2010; Wyenandt *et al.*, 2016), improve alkalinity tolerance (Colla *et al.*, 2010b; Keatinge *et al.*, 2014), raise salt and flooding tolerance (Rivero *et al.*, 2009; Martinez-Rodriguez *et al.*, 2008; Asins *et al.*, 2010; Colla *et al.*, 2010c; Savvas *et al.*, 2001; Keatinge *et al.*, 2008; Asins *et al.*, 2010; Colla *et al.*, 2010c; Savvas *et al.*, 2011; Keatinge *et al.*, 2014; Al-Harbi *et al.*, 2016) and limit the negative effect of

boron, copper, cadmium, and manganese toxicity (Edelstein *et al.*, 2007; Rouphael *et al.*, 2008b; Savvas *et al.*, 2009; Wyenandt *et al.*, 2016).

Grafting has also become a common technique for physiological studies on growth, flower induction and early flowering, as well as for the development of bioassays of virus infection or vegetative propagation (Lee and Oda, 2003; Davis *et al.*, 2008b, Flores *et al.*, 2010; Rouphael *et al.*, 2010; Geboloğlu *et al.*, 2011; Goto *et al.*, 2013; Krumbein and Schwarz, 2013; Keatinge *et al.*, 2014). In physiological studies, the recourse to grafting allows to manipulate root-sourced and shoot-sourced signals independently and has led to significant improvements of our understanding of shoot-root relationships. Root-shoot signalling is indeed important for plants because shoot and root parts experience constraints of very different nature and perform very different functions, but have to cooperate in order to successfully reach maturity and, for breeders, achieve the best possible performance.

The professional seedling producers and commercial plug seedling nurseries prefer the splice grafting method. With this method, most vascular bundles of the scion are fused with those of the rootstock and the graft union is strong enough to support all post-graft handling (Lee *et al.*, 2010). For solanaceous crops, grafting is normally made under the cotyledon and fixed with clips, elastic tube-shaped clip with side slit, or ceramic pins developed on purpose (Lee *et al.*, 2010).

Spain is currently the leading European country in using grafted vegetable transplants (129.8 million in 2009), followed by Italy (47.1 million) and France (about 28 million) (Kubota *et al.*, 2008; Lee *et al.*, 2010). About 40 - 45 million grafted seedlings were distributed in North America and in China in 2005, and ca. 20% of watermelons and cucumbers were grafted (Kubota *et al.*, 2008; Lee *et al.*, 2010). Multinational seed companies are now supplying several rootstock seeds which

virtually have little or no negative effects on fruit quality. The main grafted species for the market of food-processing industry remains the solanaceous crops (tomatoes, eggplants, capsicum peppers) and the cucurbits (watermelon, melons, and cucumbers), although many other vegetables are also grafted for other purposes (Lee and Oda, 2003).

1.2.2. Aeroponic growing method

Several hydroponic growing systems have been designed for greenhouse tomato production and these have inspired scientists in developing systems that allow the study of tomato physiology.

The standing aerated hydroponic growing method (Figure 1-1A) is the most widely used system for plant abiotic stress studies. This system is technically very simple, with plants root systems growing in a large tanks of solution, but is not suitable for large-scale commercial production (Jones, 2008; Wyenandt et al., 2016) due to the large volume of nutrient solution and the lack of recycling. A derived method is the aeroponic growing method (Figure 1-1B), in which plant roots are suspended in an empty volume in which a fine mist of nutrient solution is applied either continuously or periodically (Jones, 2008). This technique of plant cultivation has been designed for plant root system studies because it allows the contactless observation of root growth and architecture under minimal environmental restrictions (Waisel, 2002). Aeroponics is currently used in the high throughput phenotyping platform at the Université catholique de Louvain. More recently, the National Aeronautics and Space Administration (NASA) continues research on the use of aeroponic systems as a credible possibility for food production in space and microgravity environment. However, the economic value of aeroponics for the large-scale production of plants remaines to be proven (Jones, 2008).



Figure 1-1: Schematic representation of standing aerated hydroponic growing (A) and aeroponic growing methods (B) adapted from Taiz and Zieger (2010).

1.3. Chlorophyll fluorescence for assessment of nitrogen status

Optimal management of nitrogen fertilization of crops requires the use of optical methods to quickly and accurately assess the nitrogen status of aerial biomass (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). Nitrogen deficiency induced changes in concentrations of certain pigments and foliar metabolites affecting the leaf's optical properties, which can be rapidly estimated by measurements of transmittance, absorbance, reflectance or chlorophyll fluorescence. Under nitrogen deficiency, the proportion of the photons absorbed for photosynthesis, converted to chlorophyll fluorescence and dissipated in the form of heat are modified, with

13

concurrent effects on the transmitted and reflected portions of the incident radiation (**Figure 1-2**) (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). These proportions vary greatly with the chemical and physical composition of the leaf and with the light intensity absorbed. The change in leaf spectral characteristics makes it possible to establish rapid and non-destructive indicators of the nitrogen nutritional status of the aboveground biomass of a crop.



Figure 1-2: Energy budget of a typical leaf from Tremblay *et al*, 2012. PAR: photosynthetically active radiation, Indole blue-stained living section of a wheat flag leaf.

Reflectance measurements are easy to obtain, but since they are largely affected by plant biomass, their value for nitrogen content estimation is questionned (Thoren *et al.*, 2009). Transmittance measurements using the chlorophyllometer, for their part, essentially detect situations of marked nitrogen deficiency and could present a detection delay with respect to the actual onset of nitrogen deficiency (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). In contrast, chlorophyll fluorescence, despite being based on weak signals, characterizes photosynthetic activity (Buschmann, 2007) and exhibitis greater sensitivity because the fluorescence signal originates only

from the green parts of the plant (Lichtenthaler *et al.*, 1988). Chlorophyll fluorescence is also described as an early spectral signature for detecting nitrogen deficiency (Cadet, 2008). In particular, chlorophyll fluorescence induced under UV is linked to the accumulation of phenolic compounds and allows the early detection of nitrogen deficiency compared to changes in reflectance or transmittance which are based on the decrease in the chlorophyll concentration of leaves.

The use of chlorophyll fluorescence for estimating the nitrogen status of crops is based on two approaches. The first approach studies, based on Kautsky kinetics, exploits chlorophyll fluorescence as an indicator of the photosynthetic activity and stress level of plants (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). The second approach to chlorophyll fluorescence is based on the estimation of plant foliar metabolites, chlorophyll and certain phenolic compounds, which are considered as potential intrinsic indicators for the assessment of the nitrogen status of plants (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016).

Finally, other foliar pigments in relation to the nitrogen status of a culture can also be estimated by chlorophyll fluorescence which reflects phenolic and flavonoid compounds (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016).

1.3.1. Evaluation of crop nitrogen status

The emission of fluorescence is directly related to the photosynthetic activity of the plant. In particular, parameters of the variable chlorophyll fluorescence, Fv / Fm and (Fm'-F ') / Fm' provide an estimate of the PSII's photochemical efficiency (**Figure 1-3**). Since photosynthetic activity is related to nitrogen status (Evans, 1989), these chlorophyll fluorescence parameters can be used to detect nitrogen deficiencies. Under the action of stress, the photochemistry is slowed down, thus increasing

fluorescence and heat loss (Henriques, 2009). The variation of the Fv / Fm ratio as a function of nitrogen probably reflects specific responses to the environmental conditions and the nitrogen dose for the different plant species considered (Mauromicale *et al.*, 2006). The parameters of the variable chlorophyll fluorescence are not very specific because they vary according to the photosynthetic activity, which itself is sensitive to many environmental factors (*e.g.* ambient light intensity and temperature). The dynamic nature of these parameters makes their use complex and less reliable for assessing the nitrogen status of crops (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). In addition, at low luminous intensity, the Fv / Fm ratio does not respond to nitrogen deficiency (Khamis *et al.*, 1990; Ciompi *et al.*, 1996).



Figure 1-3: Variable chlorophyll fluorescence parameters from Tremblay *et al.*, 2012. F0 (minimum fluorescence level) and Fm (maximum fluorescence level) are fluorescence parameters for dark adapted leaves. Fm' (maximum fluorescence level) et F' (fluorescence level observed at time t) are fluorescence parameters for light adapted leaves.
Conversely, the parameters of chlorophyll fluorescence and foliar metabolite compounds, of a constitutive nature, vary directly according to the concentration of chlorophyll and polyphenols, are therefore more stable and can be considered for the evaluation of the nitrogen status of the crops (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). The chlorophyll fluorescence of a green leaf at room temperature is characterized by a first narrow band having a maximum emission in the red region of the spectrum around 685-690 nm (Red Fluorescence or RF) and a second band with a maximum near infrared (PIR) around 730-740 nm (Far Red Fluorescence: FRF) (Buschmann, 2007).

The chlorophyll fluorescence originates mainly from the PSII (Krause et al., 1991). Only a small part of the fluorescence in the PIR comes from the PSI. The RF and / or FRF peaks make it possible to estimate the concentration of the metabolite compounds, chlorophyll and certain phenolic compounds in leaf tissues. To estimate the latter, it is useful to consider also the FRF under excitation of UV radiation. Due to the low chlorophyll concentration of leaves, RF and FRF increase with chlorophyll content. At a higher chlorophyll contents, FRF increases slightly with chlorophyll concentration, while RF stabilizes and then decreases (Buschmann, 2007). This reduction in RF is due to the chlorophyll reabsorption of the emission spectrum of fluorescence at the wavelengths of R. Since the absorption spectrum of chlorophyll peaks in the R (of the order of 680 nm) and decreases rapidly at longer wavelengths, FRF reabsorption is low, compared to the reabsorption of RF (Buschmann, 2007). The ratio most often used in the literature is the RF / FRF ratio. This ratio decreased with the increase in chlorophyll concentration, as a result of the development of the FRF shoulder and the increased reabsorption which affects RF. Furthermore, the chlorophyll concentration of the leaf is positively correlated with its nitrogen concentration, since nitrogen is the major constituent of the tetrapyrrole nucleus of

chlorophyll. According to a broad consensus, estimating the leaf chlorophyll concentration makes it possible to estimate the nitrogen status of crop biomass and, in fine, to adjust the optimal dose of nitrogen fertilization during the season (Ben Abdallah *et al*, 2016). The RF / FRF ratio decreases with increasing nitrogen inputs (Schächtl *et al.*, 2005, Thoren *et al.*, 2009). The FRF-R / RF-R and FRF-V / RF-V ratios, induced respectively under excitation R at 635 nm (-R) or green excitation at 515 nm (-V), are also strongly influenced by the applied nitrogen doses in maize (Zhang and Tremblay, 2010). However, factors other than nitrogen were identified as affecting photosynthesis and chlorophyll content. These factors, linked to the sulfur concentration for example (Samson *et al.*, 2000) or to the water regime (Gianquinto *et al.*, 2004), show that variations in chlorophyll are not exclusively specific to the nitrogen status of the culture. This limits the use of the RF / FRF ratio, related to chlorophyll concentration, for assessing the nitrogen status of crops.

Among the phenolic compounds, flavonoids and hydroxycinnamic acids, predominantly present in the epidermis of leaves, have the property of absorbing UV radiation and thus act as a UV filter, thus protecting the mesophyll (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). The work of Cerovic *et al*. (2002) demonstrated that the ratio FRF-UV / FRF-R is proportional to the UV absorbance by the phenolic compounds present in the epidermis and therefore to their concentration. This FRF fluorescence ratio relies on the use of two excitation wavelengths, one in UV (-UV) and one in red (-R). The UV excitation (375 nm) is absorbed mainly by flavonoids (hydroxycinnamic acids), thus reducing the UV intensity reaching chlorophyll molecules in the mesophyll and decreasing the ChIF under UV excitation (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). The R excitation reaches the chlorophyll of the mesophyll without being absorbed by the phenolic compounds present in the epidermis and induces a ChIF in the PIR greater than that observed following UV excitation (Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). Cartelat *et al*. (2005)

General introduction

demonstrated a close linear relationship between the flavonoids contained in the wheat leaf extracts (measured by absorbance) and the FRF-UV / FRF-R ratio.

Moreover, the content of phenolic compounds increases with nitrogen deficiency. Chishaki et al. (1997) reported a significant increase in the concentration of phenolic compounds, such as p-coumaric acid and ferulic acid, in rice plants (Oryza sativa) affected by nitrogen deficiency. Lea et al. (2007) reported an increase in flavonoids (anthocyanins and flavonols) in the leaves of Arabidopsis thaliana caused by nitrogen deficiency. A comparison of tomato leaf extracts (lycopersicum esculentum) from a non-nitrogen deficient control with those in nitrogen deficiency showed an increase in flavonoid concentration (anthocyanidins and glycosylated flavonols) in plants deficient in nitrogen (Bongue-Bartelsman et al., 1995). The work of Stewart et al. (2001) shows that a deficiency in nitrogen makes it possible to induce an increase in flavonols. The hypothesis of carbon / nutrient balance (Coley et al., 1985), which is widely accepted, could present a physiological interpretation of the accumulation of phenolic compounds under nitrogen deficiency (Cerovic et al., 1999). This hypothesis holds that when resource availability becomes a limiting factor, plant growth is inhibited more than photosynthesis and excess carbon is thus allocated to the synthesis of phenolic compounds by stimulation of the shikimic acid pathway (Mercure et al., 2004). This situation is generally observed for plants with nitrogen deficiency (Sinclair and Vadez, 2002). Studies of Arabidopsis thaliana suggest that the synthesis of flavonoids is an adaptive response which helps the plant to cope with nitrogen decifiency (Peng et al., 2008).

According to Lillo *et al.* (2008), various environmental factors interact by influencing flavonoid concentrations in plants. Among the factors (*e.g.* nitrogen, temperature and light) studied by Løvdal *et al.* (2010), nitrogen deficiency is the most important factor contributing to the accumulation of flavonoids in tomato leaves. Sulfur

deficiency does not affect the variation of phenolic compounds (Samson et al., 2000). However, under water stress conditions, phenolic compounds increase in deficient plants (Fortier et al., 2006). Thus, the accumulation of phenolic compounds may be induced by several factors other than nitrogen, which could limit the use of the FRF-UV / FRF-R ratio in assessing the nitrogen status of crops. The indices basd on the ratio of chlorophyll and flavonoid leaf concentrations were also studied in relation to the nitrogen status of the crops. This ratio is described as a relevant indicator for the assessment of the nitrogen status of plants with a view to reasoning the input of nitrogen fertilization (Cartelat et al., 2005; Tremblay et al, 2012; Ben Abdallah et al, 2016). The [chlorophyll] / [flavonoids] ratio is a better indicator of the nitrogen status of the crop than chlorophyll alone because this ratio is independent of the leaf mass per unit area and allows to extend the range of discrimination for different levels of nitrogen fertilizers tested due to the reverse reaction of chlorophyll and flavonoids to nitrogen (Cerovic et al., 2012; Agati et al., 2013; Ben Abdallah et al, 2016). This ratio also has the advantage of attenuating, at least partially, the longitudinal heterogeneity in the leaf (Cartelat et al., 2005; Tremblay et al, 2012; Ben Abdallah et al, 2016).

1.4. The vegetative development

The vegetative development of tomato depends on the cultivar growth type. Determinate cultivars are erect and bushy with a restricted flowering and fruiting period (Jones, 2008). They grow up to 2 m in height until the stem ends. After that point, they stop producing flowers so their number of inflorescences is fixed. This group are suitable for field conditions. Siniy, Roma, Tiny Tim are some examples of determinate cultivars. At the opposite, indeterminate cultivars grows indefinitely and reach more than 12 m in 12 months in greenhouses (Jones, 2008). The

indeterminate growth is achieved by an iterative sequence in which the apical meristem evolved into an inflorescence while the axillary meristem of the leaf below the apical meristem takes over the role of the former apical meristem and produces the next stem segment. Rose de Berne, Black Cherry, Cherokee purple are some examples of indeterminate cultivars.

Semi-determinate cultivars have also been obtained. In these cultivars, the growth is determined with a late transition, or it is determined for a limited period and periodically re-enters the vegetative stage. Such cultivars are ideal for long seasons in greenhouses as they flower and fruit regularly and evenly (Jones, 2008). San Marzano, Celebrity, Flamenco are some examples of tomato cultivars for this type of growth.

1.4.1. Shoot architecture of tomato

The shoot architecture of tomato varies from vines which spread horizontally, to more bushy types. The stem is between 0.5 at 4 cm in diameter at the base and is covered with glandular and non-glandular hairs. At the top of main stem is the apical meristem, an active cell division region where new leaves and flowers initials are formed. Leaves are arranged alternatively along of the stem with a phyllotaxy of $4\pi/5$ radians (or an angle of 144 degrees) (Najla *et al.*, 2009) and are produced sequentially with a phyllochron of ca. 0.029 (°C.day)-1 (Najla *et al.*, 2009).

The shoot system architecture of the tomato plant displays a branching pattern. The activity of the terminal bud produces new stem segments and new axillary buds from which branch stems can grow. During vegetative growth, the growth of lateral branches is somewhat inhibited due to the apical dominance. Lateral branches grow

out when the terminal bud stops growing, is cut off by pruning, or evolves in an inflorescence.

The leaf of tomato is compound with a large terminal leaflet and up to 8 large lateral leaflets, which may themselves be compound. The leaves are covered with hairs of the same types as on the stem. The leaflets are usually petiolate and irregularly lobed with toothed edges. The leaflets are initiated in basipetal progression from the terminal leaflet towards the leaf-stem junction (Picken *et al.*, 1986; Shwartz *et al.*, 2016).

1.4.2. Root growth, development and architecture

Roots play several key roles: anchorage, water and soil nutrients uptake and transport to the stem, accumulation of reserves, support of complex symbiotic associations with the microorganisms, modification of soil structure (Gregory, 2006; Reubens *et al.*, 2007; Danjon and Reubens, 2008, Hodge *et al.*, 2009; Bellini *et al.*, 2014). These functions are strongly conditioned by root architecture, *viz.* the three dimensional structure of the roots, which includes topological and geometric features (Gregory, 2006; Reubens *et al.*, 2007; Danjon and Reubens, 2007; Danjon and Reubens, 2008).

The backbone of the tomato root system is made of the primary root present in the embryo, the adventitious roots formed at the base of the stem and the lateral roots formed on primary, adventitious and other lateral roots. Several processes are involved in the construction of the root system: branching, senescence, axial and radial growth and tropism (Hodge *et al.*, 2009; Orman-Ligeza *et al.*, 2013; Bellini *et al.*, 2014; Orman-Ligeza *et al.*, 2014). The production of adventitious and lateral roots is coordinated at the local and the plant level and displays a strong plasticity that is achieved through complex developmental pathways (Orman-Ligeza *et al.*, 2013).

Root growth, also called "axial growth", is the outcome of cell division and expansion which occur in the root meristem and push the meristem ahead. It contributes to the colonization of new volumes of soil. The root meristem comprises a distal meristem that gives rise to the cap, a central zone with a reduced mitotic activity, the quiescent center and a division zone which produces all cells making up the different primary tissues (Orman-Ligeza *et al.*, 2013; Bellini *et al.*, 2014; Orman-Ligeza *et al.*, 2014). Proximal to the division zone is the elongation zone in which cells expand rapidly to reach their final size. Secondary growth, also called "radial growth", occurs on some root types and leads to a diameter increase. It is due to the activity of a secondary meristem, cambium, formed in the root maturation zone.

The individual root growth rate usually correlates with the apical meristem diameter (Pagès, 1995; Lecompte *et al.*, 2005) which, in turn, correlates with growth duration, respiratory activity and gravitropism (Orman-Ligeza *et al.*, 2013; Bellini *et al.*, 2014; Orman-Ligeza *et al.*, 2014). Other factors also influence the root growth rate, such as temperature, soil compaction and mineral or organic toxicities. For example, soil compacity reduces water content and oxygen concentration, leading to a restriction of the root elongation.

The direction of root growth has a large influence on the shape of the root system and on the volume of soil that is explored (Coutts, 1983). The main factor affecting the direction of the roots is gravitropism, a phenomenon by which different parts of a plant are directed at a specific angle relative to the gravity vector (Orman-Ligeza *et al.*, 2013; Bellini *et al.*, 2014; Orman-Ligeza *et al.*, 2014). Primary and adventitious roots often display a positive gravitropism and tend to grow vertically into the soil, while lateral roots display a plagiotropism and tend to penetrate the soil obliquely or horizontally. As a result, in field conditions, the root system of tomato extends horizontally to 1.5 m from the plant and to a somewhat shorter depth (Picken *et al.*, 1986). The primary seminal or taproot may grow deeper than 0.5m, although it is often damaged in culture (Picken *et al.*, 1986). The growth direction can also be modified by other environmental factors such as temperature, humidity and the concentration of mineral elements. Hydrotropism, chimiotropism and oxytropism have been demonstrated and the molecular mechanisms underlying them is being uncovered (Heller, 2004; Taiz and Zeiger, 2010).

The root branching mechanism differs from shoot branching. Lateral roots are initiated from two pericycle cells which acquire their founder identity after their differentiation as pericycle cells (Barthélémy and Caraglio, 2007). After several rounds of cell divisions which establish the new stem cells of the lateral root, a meristem will be formed and primordia elongate through the parent root cortex (Raven *et al.*, 2005). Root branching is strongly sensitive to soil heterogeneity (*e.g.* hydropatterning).

Finally, root senescence plays an important role in the dynamics of soil root occupation. Root senescence is controlled by several factors, including carbon allocation to roots, nitrogen nutrition and the presence of mycorrhizae (King *et al.*, 2002; Hodge *et al.*, 2009; Kraiser *et al.*, 2011). It can also be induced by an environmental pressure, like the presence of pathogenic fungi, anoxia, the presence of pollutants and many other factors outside the plant.

1.5. Mineral nutrition of tomato

The maintenance of an adequate supply of nutrient under heterogeneous and fluctuating environmental conditions is one of the key challenge of plant nutrition in agriculture and horticulture (Kraiser *et al.*, 2011). In addition, the response of crops,

including tomato, to a particular nutrient can change with cultivar, cultural practices, substrate and environmental conditions (Bellini *et al.*, 2014). The plant nutrition involves 16 essential nutrients but also many other chemical elements that can have beneficial or harmful effects on plant metabolism and have to be considered (Kraiser *et al.*, 2011, Bellini *et al.*, 2014). A primary aim of fertilization is to provide the crop with nutrients in quantities that are optimal for their availability throughout the season. Nitrogen is the most abundant mineral element in plant tissues. For this reason, it is the mineral nutrient required in the largest amount and its availability is a major factor limiting plant growth in agricultural as well as in natural environments (Galloway and Cowling, 2002; Marschner, 1995; Epstein and Bloom, 2005; Foyer and Zhang, 2010; Wyenandt *et al.*, 2016).

Nitrogen deficiency, even temporary, usually leads to significant yield reductions, while nitrogen inputs in excess to the needs of crops lead to a reduction in nitrogen use efficiency (Goffart *et al.*, 2013). In fact, nitrogen enters in the composition of indispensable molecules such as amino acids and nucleic acids but also in that of certain secondary metabolites. This element represents 1.2 to 7.5 g per 100 g of dry matter in plants (Meyer *et al.*, 2008; Foyer and Zhang, 2010). In tomato, the nitrogen contents in the plant depend on the organs. The vegetative parts (roots, stems and leaves) are the richest nitrogen compartments and contain about 6 g of nitrogen per 100 g of dry matter, while fruits contain only 1.7 g of nitrogen per 100 g of dry matter (Toor *et al*, 2006; Foyer and Zhang, 2010).

1.5.1. Nitrogen in plants ecosystem

Nitrogen is present in the biosphere in various chemical forms. N_2 represents around 80% of the atmosphere composition but cannot be used directly by plants

(Sanhueza, 1982; Foyer and Zhang, 2010). It enters the biological nitrogen cycle by prokaryotic conversion of N₂ to ammonia, lightning and photochemical conversion of N₂ to nitrate by atmospheric and industrial fixation (Marschner, 1995; Foyer and Zhang, 2010). After nitrogen fixation, nitrate or ammonia can be assimilated by plants, go through biochemical processes that revert them back to N₂, or be assimilated for the biosynthesis of small nitrogen-containing metabolites (amino acids, urea, small polypeptides) which can be released back to the environment by secretion, excretion, or by the decomposition of organic matter (Marschner, 1995; Foyer and Zhang, 2010; Kraiser *et al.*, 2011).

The particularity of nitrogen is that it is the only nutrient that can be supplied to plants in both anionic (NO3⁻) and cationic (NH4⁺) form (Forde and Clarkson, 1999; Foyer and Zhang, 2010; Fukushima and Kusano, 2014). Thereby, the total cation to anion uptake ratio will be strongly influenced by the fraction of ammonium to the total nitrogen supply (Imas *et al.*, 1997; Savvas *et al.*, 2006; Foyer and Zhang, 2010). The change in the cation/anion are electrochemically compensated by alterations in the protons (H⁺) flux or basic anions in the root zone, which will influence significantly the pH of rhizosphere (Barber, 1984; Lea-Cox *et al.*, 1996; Imas *et al.*, 1997; Foyer and Zhang, 2010). Because the uptake of nutrients is influenced by the pH of the external medium, the form of nitrogen supply can induce nutritional disorders in plants (Islam *et al.*, 1980; Imas *et al.*, 1997; Adams, 2002; Foyer and Zhang, 2010). The form of nitrogen for plants may also influence the uptake of other nutrients due to ion antagonism (Marschner, 1995) as well as plant metabolism, due to differences in the intracellular assimilation pathways (Raab and Terry, 1994; Gerendás *et al.*, 1997; Foyer and Zhang, 2010).

The first studies on the effects of nitrogen source on tomato and its interactions with other nutritional and environmental factors concluded that tomato is sensitive to the provision of ammonium as a sole or dominating nitrogen form (Kirkby and Knight, 1977; Ganmore-Neumann and Kafkafi, 1980; Magalhães and Wilcox, 1983; Errebhi and Wilcox, 1990; Imas *et al.*, 1997). In the studies of Siddiqi *et al.* (2002) and Akl *et al.* (2003), a range between 0.15 - 0.25 of the total-N supply was associated with low pH levels (<5) in the root zone and a reduction of vegetative growth and fruit yield. In other studies, however, the total dry weight and fruit dry weight were still increasing when the ammonium fraction was 0.25 (Claussen, 2002; Dong *et al.*, 2004). It is important to note that Claussen (2002) maintained the rhizosphere pH above 6, unlike to Siddiqi *et al.* (2002) and Akl *et al.* (2003). Therefore, it seems that the effect of ammonium is principally driven by its impact on the rhizosphere pH (Chaignon *et al.*, 2002).

Plants have evolved two types of uptake system for nitrate and ammonium (Crawford and Glass, 1998; Ludewig et al., 2007; Foyer and Zhang, 2010): a low affinity transport system (LATS) which operate at high nutrient concentrations (>1 mM) and a high affinity transport system (HATS) which operate at low nutrient concentrations (<1 mM) (Kraiser et al., 2011; Bellini et al., 2014). The changes in the pattern of growth and development in coordination with a modulation of HATS and LATS function allows plants to cope with heterogeneous and fluctuating N availability in the soil (Robinson, 1994; Zhang and Forde, 2000; Lopez-Bucio et al., 2003; Zhang et al., 2007; Vidal and Gutierrez, 2008; Forde and Walch-Liu, 2009; Vidal et al., 2010a; Kraiser et al., 2011). In addition, plants can also interact and associate with many microorganisms that may also contribute to an adequate supply N in natural environments (Gage, 2004; You et al., 2005; Kraiser et al., 2011). Soil organic compounds can also contribute to the plant nitrogen nutrition (Lipson and Näsholm, 2001; Näsholm et al., 2009). The soil comprises a pool of low molecular weight dissolved organic N in the soil (chain of mail amino acids). This pool is largely dynamic because amino acids are quickly taken up by plants and microorganisms. Finally, urea

excreted by many organisms or applied as fertilizer is also an abundant source of nitrogen available in the soil (Kraiser *et al.*, 2011). It has also been shown that proteins may be used by plants as N source without obvious assistance from other organisms (Paungfoo-Lonhienne *et al.*, 2008). The significance of proteins for plant nutrition remains, however, to be established. Nitrogen from soil proteins could be used via the degradation of proteins by proteases present in root exudates, but it is not excluded that root could absorb intact proteins from the soil by means of unknown transporters or by endocytosis (Paungfoo-Lonhienne *et al.*, 2008).

1.5.2. Nitrogen nutrient uptake systems

Nitrate is the preferred form of plants, but nitrate anions (NO3⁻) will be converted during their assimilation into ammonium (NH4⁺) ions, which will be incorporated into organic molecules (Masclaux-Daubresse *et al.*, 2010). Once nitrate is absorbed by roots, it can either be immediately reduced, or exported to the leaves via the xylem, or stored in the vacuoles or discharged into the external environment. The conduction of the nitrate to the xylem vessel is effected by osmosis, according to the concentration gradient, and by the symplastic way (Masclaux-Daubresse *et al.*, 2010). Then the vertical transit to the leaves is assured by the rise of the raw sap, following a gradient of decreasing water potential (Meyer *et al.*, 2008; Masclaux-Daubresse *et al.*, 2010; Foyer and Zhang, 2010; Fukushima and Kusano, 2014). The distribution of the reductive assimilation of nitrates between the roots and the aerial parts varies. In many herbs, nitrate reduction occurs mainly in the leaves, whereas it occurs in roots in woody plants (Grignon *et al.*, 1997; Foyer and Zhang, 2010; Fukushima and Kusano, 2014). This distribution also depends on environmental conditions and, in particular, on the nitrate concentration of the medium.

In fact, when the nitrate concentrations are low, the reduction takes place in roots and the leaves will receive nitrogen in the organic form. On the other hand, if the concentration is greater, a stream of nitrate can reach the leaves. The reduction of nitrates is performed by two enzymes: nitrate reductase (NR) and nitrite reductase (NIR). NR converts NO3⁻ into NO2⁻ into the cytoplasm. NIR reduces NO2⁻ to NH4⁺ in plastids (chloroplasts) (**Figure 1-4**).

The reduction of one mole of NO3⁻ to NH4⁺ releases one mole of OH⁻ and increases the pH inside cells. In the leaves, this alkalization is neutralized by the biosynthesis of organic acids, mainly malic acid. In the roots, these ions can be released into the soil via an ion exchange system (Foyer and Zhang, 2010; Fukushima and Kusano, 2014). NR and NIR are subject to numerous regulations, including factors of the environment, such as light or nitrate concentrations, endogenous factors such as circadian rhythm, or hormone application (*e.g.* cytokinin and ethylene) (Masclaux-Daubresse *et al.*, 2010; Foyer and Zhang, 2010; Fukushima and Kusano, 2014). The assimilation of nitrate also requires electron donors (NADH, NADPH and ferredoxin) and energy which are brought about by the reactions of photosynthesis, respiration, and by way of the pentose phosphates (Masclaux-Daubresse *et al.*, 2010). These same reactions provide carbon skeletons used in the assimilation of ammonium which corresponds to the integration of this ion in carbon chains to form organic molecules (**Figure 1 4**).

In cells, ammonium may have several origins: it can be taken directly from the medium (HATS system and LATS), resulting from the reductive assimilation of NO3⁻, or derived from photorespiration. The enzymes responsible for the assimilation of ammonium into organic compounds are present in several organs of the plant. In the higher plants, the assimilation of ammonium mainly involves two enzymes:



Figure 1-4: Schematic presentation of key enzymes involved in nitrogen management in (A) young and (B) senescing leaves from Masclaux-Daubresse *et al* (2010). (A) Nitrate reductase (NR) and asparagine synthetase (AS) are localized in the cytosol, and nitrite reductase (NiR), glutamine synthetase 2 isoenzyme (GS2), glutamate synthase (GOGAT) and carbamoylphosphate synthetase (CPSase) within the plastids of mesophyll cells. Glutamine synthetase isoenzyme 1 (GS1) and AS are located in the cytosol of companion cells. (B) Senescence-associated events include chloroplast degradation and translocation of plastid proteins to the central vacuole via senescence-associated vacuole (SAV) trafficking. Amino acid recycling occurred in mitochondria and cytosol of mesophyll cells and companion cells. Glutamate dehydrogenase (GDH), GS1 and AS are the major enzymes involved in the synthesis of glutamine, glutamate and asparagine in the phloem.

General introduction

glutamine synthetase (GS) and glutamate synthase (or glutamine 2-oxoglutarate aminotransferase, GOGAT). The ammonium ion is first incorporated into glutamate during a reaction catalyzed by GS to form glutamine (Masclaux-Daubresse *et al.*, 2010). Then, under the action of GOGAT and in the presence of α -cetoglutarate, two glutamate molecules are formed, one of which will be recycled for the synthesis of glutamine (Masclaux-Daubresse *et al.*, 2010; Foyer and Zhang, 2010; Fukushima and Kusano, 2014). The nitrogenous groups of glutamine and glutamate are then transferred to other molecules to form the various nitrogen compounds of the cell, including the amino acids. The other assimilation pathway involves glutamate dehydrogenase (GDH) which allows the formation of glutamate, however this reaction is mostly observed in vitro or in bacteria and fungi. In higher plants the role of GDH is still being determined (Masclaux-Daubresse *et al.*, 2010). This enzyme also works in the direction of catabolism of glutamate, releasing NH4⁺ and carbon skeletons (Masclaux-Daubresse *et al.*, 2010). These carbon substrates could compensate for a decrease in photosynthetic activity.

Plants can therefore take nitrogen in the form of nitrate or ammonium, and the form of nitrogen provided changes the growth and development of plants. It should also be noted that in plants the nitrogen and carbon metabolisms are interdependent. The assimilation of nitrogen by the plant requires energy and reducing power that are produced during respiration and photosynthesis. Conversely, photosynthetic assimilation of CO2 requires the development of nitrogen molecules such as enzymes (Masclaux-Daubresse *et al.*, 2010; Foyer and Zhang, 2010; Fukushima and Kusano, 2014). Thus any modification of the nitrogen metabolism is likely to affect the assimilation and the metabolism of the carbon, and *vice-versa*.

1.6. Root architecture responses to nitrogen nutrition

Plant adjust their root architecture as a function of the availability and spatial distribution of macronutrients and micronutrients and of their developmental stage. The responses to phosphorus and nitrogen limitations attract much attention from researchers for essentially two reasons: they are major plant nutrients and they represent contrasting models of limiting resources. Phosphorus has a low availability and mobility in the soil, because its dominant form is inorganic phosphate which forms insoluble complexes with cations, particularly aluminum, iron and calcium (Bellini *et al.*, 2014). On the contrary, the nitrogen has a high availability and is very mobile in the soil.

In many species, localized nitrate supply induces a rapid proliferation of roots in the nitrogen patch, which involves changes of growth and branching (Drew *et al.*, 1973; Drew and Saker, 1975; Robinson, 1994; Hodge, 2004; Little *et al.*, 2005; Remans *et al.*, 2006a; Walch-Liu *et al.*, 2006a; Gifford *et al.*, 2008; Walch-Liu and Forde, 2008; Vidal *et al.*, 2010b). This localized architectural response is presumably an adaptation to ensure the efficient acquisition of a mobile and essential element such as nitrate in communities of competing plants (Robinson, 1994; Robinson, 1996; Robinson, 2001; Hodge, 2004). The mechanisms involved are not yet fully understood, but involves alterations of primary root growth (Walch-Liu *et al.*, 2006b; Walch-Liu and Forde, 2008; Vidal *et al.*, 2010a), lateral root initiation (Little *et al.*, 2005; Remans *et al.*, 2006b; Gifford *et al.*, 2008) and lateral root elongation (Zhang and Forde, 1998; Zhang *et al.*, 1999; Vidal *et al.*, 2010a). The regulation of root architecture by nitrate is complex as, in high nitrogen conditions, the local response to nitrate heterogeneity gives way to systemic inhibition of primary and lateral root elongation.

The first regulatory factor involved in modulating root architecture in response to a localized nitrate supply was the nitrate-inducible MADS-box transcription factor,

General introduction

NITRATE REGULATED 1 (ANR1) (Zhang and Forde, 1998; Zhang et al., 1999; Malamy and Ryan, 2001). This regulatory factor was found in a reverse genetic screen designed to isolate genes whose expression was induced in nitrate rich patches and forms part of the MADS-box transcription factor gene family. A decreased root growth response to a localized nitrate supply was showed with the help of transgenic plants in which the ANR1 was repressed. The nitrogen signaling pathway comprises NRT1.1 and NRT2.1, whose role in the modulation of root system architecture has also been demonstrated by reverse genetics approaches (Zhang et al., 1999). The NRT1.1 mutant plants shows a strong inhibition of the root colonization of nitraterich patches (Remans et al., 2006a). NRT1.1 is induced by nitrate and by the phytohormone auxin (Munõs et al., 2004) and operates as an auxin influx facilitator whose activity depends on nitrate concentration (Krouk et al., 2010). In low nitrogen environment, NRT1.1 promotes auxin transport out of the lateral root primordium and represses lateral root development (Krouk et al., 2010). The lateral root initiation control in response to low nitrate supply is also mediated in part by NRT2.1 that causes the repression of lateral root initiation when the carbon/nitrogen ratio is high (Malamy and Ryan, 2001; Little et al., 2005; Remans et al., 2006a).

Other signaling molecules derived from nitrogen metabolism contribute to adjustments of root system architecture. Nitric oxide is involved in the regulation of primary root elongation and lateral root initiation in tomato plants (Correa-Aragunde *et al.*, 2004; Costigan *et al.*, 2011). In maize, a reduction in nitric oxide content in the apical cells inhibits root growth in relationship with endogenous auxin (Tian *et al.*, 2008; Zhao *et al.*, 2007). Nitric oxide is also involved in auxin induced adventitious root development in cucumber (*Cucumis sativus*) (Pagnussat *et al.*, 2003; Pagnussat *et al.*, 2004). The formation of new adventitious roots is regulated

by cyclic guanosine monophosphate-dependent and -independent signaling pathways (Pagnussat *et al.*, 2003; Pagnussat *et al.*, 2004).

The direct impact of nitrogen nutrition on adventitious root formation still needs to be investigated, but the carbon/nitrogen ratio has been pointed as influencing both nutritional status and adventitious root formation (Bellini *et al.*, 2014). Carbon and nitrogen metabolisms and intimately connected and modifications in nitrogen supply can influence carbon assimilation, allocation, and partitioning within plants. For example, in *Pelargonium* spp., the carbon/nitrogen ratio affects the adventitious root formation in future cuttings (Druege *et al.*, 2004). After storage under low temperature and light, high nitrogen supply and high light conditions lead to an increase of endogenous nitrogen content, which has a positive effect on the rooting of future cuttings (Druege *et al.*, 2004). However, this response is conditioned by carbohydrate availability as high nitrogen has no effect or even inhibits adventitious root formation when endogenous sugar content is low (Druege *et al.*, 2004; Zerche and Druege, 2009).

Other physiological mechanisms also condition the overall growth response to nitrogen supply (Kraiser *et al.*, 2011). In particular, there is evidence that some alterations of root growth are the outcome of a cascade of events initiated in the shoot (Giuliani *et al.*, 2005; Sergeeva *et al.*, 2006). The classical dichotomy between root and shoot research may have contributed to our limited understanding of these whole plant regulatory processes.

Chapter 2

Objectives and outline

2.1. Rationale of the thesis

The main objective of this thesis is to evaluate the genetic determinism of root system architecture (RSA) and its response to nitrogen deficiency in tomato (S. *lycopersicum*). It is expected that the information gained from the thesis will help disentangling which components of RSA contribute to plant performance under nitrogen deficiency.

An innovative strategy was used, that exploits the ease of grafting in tomato. Grafting is a surgical technique that allows manipulating shoot and roots independently. It was used here to assess the effect of root traits on crop performance. The same scion (*Solanum lycopersicum* L. 'Boludo F1', 'Monsanto') was grafted onto 144 different rootstocks: six accessions from S. *lycopersicum* (*'Cerasiforme'*) and S. *pimpinellifolium*, selected for drought tolerance (sourced from The World Vegetable Center, AVRDC); nine introgression lines from S. *lycopersicum* × S. *pennellii* and × S. *habrochaites*, selected for high root/shoot ratio, salinity and drought tolerances (sourced from The Tomato Genetics Resource Center, TGRC); a population of 129 recombinant inbred lines (RILs) derived from a salt sensitive genotype of S. *lycopersicum* var. *cerasiforme* and a salt tolerant line from S. *pimpinellifolium* L. (Monforte *et al.*, 1997) that had been developed at Instituto Valenciano de Investigaciones Agrarias (IVIA) to enable the genetic dissection of

35

quantitative traits. During this thesis, this material was phenotyped for RSA and plant growth under low and high nitrogen and the genetic analysis was carried out. This work was part of the EU-FP7 project RootOPower (2012-2015).

The research strategy was organized around three main questions:

- i) Can we design high throughput phenotyping tools to monitor RSA, plant growth and nitrogen status under nitrogen deficiency?
- ii) What are the genetic relationships between root system morphology and biomass production under nitrogen deficiency?
- iii) Can we identify traits that improve plant growth under nitrogen deficiency?

2.2. Outline of the thesis

The thesis document comprises four experimental chapters (Ch. 3 to 6) (Figure 2-1) organized in two parts. The first part (Ch. 3 and 4) relates to the development of nitrogen monitoring methodologies, while the second part (Ch. 5 and 6) reports the genetic dissection of RSA and growth under low and high nitrogen (Figure 2-1). The experimental chapters are followed by a general discussion and the presentation of a few perspectives for future research (Figure 2-1).

In chapter 3, we report the development of a low-cost, high throughput method to measure plant nitrogen content, based on Near-infrared microscopy (NIRM). This novel method allows the determination of the nitrogen content in reduced sample volumes of tomato leaf powder.

Chapter 4 is devoted to the assessment of optical methods to monitor nitrogen content evolution in tomato. These methods were calibrated using NIRM. A special emphasis was given to test the possibility of transforming optical indicator values into absolute values of nitrogen content.







Figure 2-1: Schematic outline of the thesis.

In chapter 5, we report the results of a genetic dissection of root system architecture (RSA) and plant growth under low and high nitrogen and we analyze the genetic relationships between the traits of this two conditions.

The last experimental chapter (Ch. 6) is devoted to develop a preliminary model attempt to identify the main strategies (*i.e.* RSA traits) that confer crop performance under nitrogen deficiency in this segregating population.

In chapter 7, we summarize the main results and present some perspectives for future research to enhance agronomic stability and sustainability under abiotic stresses.

Due to the presentation of the chapters in a publication format in this thesis, a number of repetitions could not be avoided.

Chapter 3

Determination by near infrared microscopy of the nitrogen and carbon content of tomato (*Solanum lycopersicum* L.) leaf powder

In this chapter, a near infrared microscopy (NIRM) method was developed to determine the nitrogen content in tiny amounts of tomato (*Solanum lycopersicum* L.) leaf powder and quantify the response of tomato to low nitrogen supply. The main benefits of this technique compared to conventional methods (*e.g.*, the Kjeldahl method, the Dumas method and NIRS) is essentially the simplicity of the sample preparation procedure, the small analytical costs and times, and the small amount of tissue required.

This chapter was previously published as:

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39

Abstract

Near infrared microscopy (NIRM) has been developed as a rapid technique to predict the chemical composition of foods, reduce analytical costs and time and ease sample preparation. In this study, NIRM has been evaluated as an alternative to classical chemical analysis to determine the nitrogen and carbon content of small samples of tomato (Solanum lycopersicum L.) leaf powder. Near infrared spectra were obtained by NIRM for independent leaf samples collected on 216 plants grown under six different levels of nitrogen. From these, 30 calibration and 30 validation samples covering the spectral range of the whole set were selected and their nitrogen and carbon contents were determined by a reference method. The calibration model obtained for nitrogen content proved to be excellent, with a coefficient of determination in calibration (R²c) higher than 0.9 and a ratio of performance to deviation (RPDc) higher than 3. Statistical indicators of prediction using the validation set were also very high (R²p values > 0.90). However, the calibration model obtained for carbon content was much less satisfactory (R²c < 0.50). NIRM appears as a promising and suitable tool for a rapid, non-destructive and reliable determination of nitrogen content of tiny samples of tomato leaf powder.

3.1. Introduction

For four decades, the food sector has adopted near-infrared (NIR) based techniques for the quantitative control of raw materials and final products. These techniques offer the possibility to investigate simultaneously physical, biological and nutritional features of complex matrices, require small sample amounts and simple small preparation, and involve small analytical costs and times (Cozzolino, 2009; Bittner et al., 2013; Krämer et al., 2013; Barbin et al., 2014; Baeten et al., 2015; Krämer et al., 2015). The number of NIR or mid-infrared (MIR) applications is continuously increasing. The high sample throughput of these techniques have been used to predict several qualitative and quantitative features of fruits, vegetables, grains, oils, tea and other agricultural products, as substitute or complement of conventional destructive methods (Cozzolino, 2009; Dale et al., 2011; Tuccio et al., 2011, Alander et al., 2013; López et al., 2013; Barbin et al., 2014; Pojić et al., 2015; Wang et al., 2014; Xin et al., 2015). In particular, they have proven as fast and routinely applicable alternatives to conventional methods of quantification of the total protein content, e.q. the Kjeldahl method (total organic nitrogen content), the Dumas method (total nitrogen content) and spectroscopy (infrared absorbance of proteins) (Schulz and Baranska, 2007; Baranska and Schulz, 2009; Krämer et al., 2013; Krämer et al., 2015).

NIR spectra of food products include mainly absorption bands characteristic of O–H, C–H, N-H, S-H and C-C groups. These bands are the result of the interaction between photons and matter. These interactions occurring in the NIR region of the electromagnetic spectrum induce vibration transitions in the second, third or higher excited states or overtones, as well as combinations derived from fundamental vibrations that occur in the MIR region (Baeten and Dardenne, 2002; Westad *et al.*, 2008; Krämer *et al.*, 2015;). NIR spectra quality is impacted by various factors, such as the physical state of the product (solid or liquid), temperature of the sample, granulometry (*e.g.* powder or non-concrete products), homogeneity and presence of impurities (Baeten *et al.*, 2015).

In plant science, NIR-based methods offer the possibility to identify and quantify primary and secondary metabolites without preliminary physical separation. Barbin *et al.* (2014) and Krähmer *et al.* (2015) have recently assigned the most characteristic NIR bands of some primary (*e.g.* carbohydrates, lipids, proteins) and secondary (*e.g.* phenolic substances, terpenoids, alkaloids) metabolites. One of the main limitations in plant research remains the need to harvest sufficient amounts of material, typically for experiments with Arabidopsis or for phenotyping experiment aiming at mapping metabolites in an organ. The combination of near-infrared spectrometry (NIRS) with microscopy appears to be a viable solution to address this challenge. With this technique, the NIR spectra of a sample area as small as 1 μ m² can be collected non-destructively (Baeten *et al.*, 2015).

NIR microscopy (NIRM) is a relatively novel technique that enables the spectral analysis of individual particles. NIRM was first used in feed analysis to detect forbidden animal protein in compound (Piraux and Dardenne, 1999; Baeten and Dardenne, 2001). Many studies have demonstrated the value of NIRM for producing high-quality spectra from small particles (< 500 μ m) (Baeten *et al.*, 2005; de la Haba *et al.*, 2007; de la Roza-Delgado *et al.*, 2007; Fernández-Ibáñez *et al.*, 2009; Pérez-Martin *et al.*, 2009; Lü *et al.*, 2011; Yang *et al.*, 2011; Boix *et al.*, 2012; Fernández *et al.*, 2013). Because NIRM results can be shared easily in networks of laboratories (Fernández *et al.*, 2013), the technique has been validated at European level (Boix *et al.*, 2012).

This pioneering study evaluates the value of NIRM to predict the nitrogen and carbon contents (*e.g.*, main primary plant metabolites) in tiny samples (< 40 mg) of tomato leaf powder.

3.2. Material and Methods

3.2.1. Plant material and growth conditions

The tomato (*Solanum lycopersicum* L.) variety Ailsa Craig was used in this study. The experiment was conducted in Louvain-la-Neuve, from 23 July 2013 to 12 September 2013. Seeds were surface-sterilized by soaking in a 5 % (v/v) sodium hypochlorite solution for 15 min and rinced three times with deionized water. Seeds were germinated in a loam substrate incubated in a growth chamber (24 °C / 22 °C; 80 % RH; 16 h photoperiod; 150 μ mol.m-2.s-1 PAR). Ten days after sowing, derooted tomato were washed with deionized water and transferred individually in 1.45 L pots filled with a mix of perlite and vermiculite (50/50).

After seven days of rooting in the growth chamber, the tomato were transferred in a greenhouse for seven days acclimation period. A data-logger (TinyTag Ultra, model TGU-1500, INTAB Benelux, Netherlands) was used to record climate data during the experiment; Tmean 26.5/18.2 °C day/night, (max. 34.8/27.9 °C day/night, min. 13.1/12.8 °C day/night) and RHmean 52.8/69.0 % day/night (max. 93.5/96.4 % day/night, min. 27.8/41.6 % day/night). The photoperiod was set at 16 h and the solar radiation was supplemented with Philips HPLR lamps (400 W) providing 40 μ mol m-2 s-1 at the canopy level. During these periods, plants were watered three times per week using a modified Hoagland solution (Hoagland and Arnon, 1950) with a nitrogen concentration of 13 mmol.I-1 (**Table 3-1**). The set of plants was then splitted into six groups of 12 plants which were exposed to one of six nitrogen concentrations (13.0; 6.50; 3.25; 1.63; 0.81; 0.41 mmol.l-1) (**Table 3-1**). They were watered three times per week with a volume of 100 ml solution from the top of the pot.

Elements	Solutions					
[Total nitrogen] (in mmol.l ⁻¹)	13	6.50	3.25	1.63	0.81	0.41
NO3⁻ (in mmol.l⁻¹)	12.00	6.00	3.00	1.50	0.75	0.38
NH4 ⁺ (in mmol.l ⁻¹)	1.00	0.50	0.25	0.13	0.06	0.03
SO4 ²⁻ (in mmol.l ⁻¹)	1.00	3.75	5.13	5.82	6.16	6.33
H2PO4 ⁻ (in mmol.l ⁻¹)	2.01					
K+ (in mmol.l ⁻¹)	6.01					
Ca ²⁺ (in mmol.l ⁻¹)	3.50					
Mg ²⁺ (in mmol.l ⁻¹)	1.00					
рН	5.6					
EC (dS/m)	1.60	1.51	1.53	1.51	1.51	1.50

Table 3-1. The principal chemical compounds (in mmol.l⁻¹) with the different electrical conductivity (EC in dS/M) and pH used in nitrogen solutions (1.3; 6.50; 3.25; 1.63; 0.81; 0.41 mmol.l⁻¹) achieve from a modified Hoagland solution.

From the start of the treatment application, three plants were harvested weekly in each treatment. The shoot and root parts were dried by oven-drying at 65 °C until constant weight. Finally, dry aerial parts were crushed with a sample mill (CT 193 Cyclotec[™], Foss, Hillerød, Denmark) to obtain a powder (with a dry matter weight between 0.01 and 2.41 g).

The complete experiment was performed in three simultaneous full repetitions, generating 216 samples in total.

3.2.2. NIR microscopy

The near infrared analyses were performed using a completely automated Fourier Transform-IR imaging Microscope (Hyperion 3000, Bruker Optics, Ettlingen, Germany). Data were recorded in the range from 9.000 to 4.000 cm-1 with a spectral resolution of 8 cm-1.

All spectra were collected with 32 co-added scans. Vibrational spectroscopy was performed directly on crushed shoot powder. For each sample, 10 spectra were collected at different spatial location of the samples spread on an aluminum plate with 96 wells containing the sample allocated in 2 or 5 wells, based upon the dry weight available. After the analysis of the sample, the file including the 10 spectra collected was opened in the Opus 6 (Bruker Gmbh, Germany) to verify the presence of the characteristic NIR bands and the absence of noisy spectra.

One of the samples was analyzed several times during the three days of measurement to determine the value of inter-day reproducibility. The subsequent chemometric evaluation has exclusively been based on the average spectra on all samples. **Figure 3-1** shows the workflow of the analysis process by NIRM.



Figure 3-1. Workflow of the NIRM analysis performed. First step is the reduction of leaf (a) to powder (b) and after this powder is spread into the 96 well plate (c). The plate is then presented to the microscope (d) and 10 NIR spectra are collected at different locations (e).

Thirty samples of the total set (216) have been used to construct the model (calibration set) and thirty others to validate the model (validation set). The

calibration and validation sets analyzed were selected to cover the full range of NIR spectral variation.

3.2.3. Reference analysis

The nitrogen (N-value in %) and carbon (C-value in %) content of each sample of the calibration and validation sets were determined by combustion according to the Dumas method using 5 mg of powder. The analysis was carried out on an elemental analyzer (Flash EA 1112 series, Thermo Finnigan, San Jose, CA, USA). The time interval separating the measurements of these two sets was three months during which the samples were stored in hermetic pots conserved in dark room. The calibration curves for the elemental analyzer were determined by using atropine standard to different known concentration of carbon and nitrogen contents and routinely checked using this standard. Six samples from the calibration sets were measured in duplicate (the second analysis was performed at the same time as the analysis of the validation set). This allowed to check the stability and reproducibility of the reference method and estimate the error of the elemental analyzer.

3.2.4. Statistical analysis

Multivariate chemometric analysis was performed using the Unscrambler[®] X software version 10.3 (Camo Inc., Oslo, Norway) and in accordance with the considerations formulated by Dardenne (2010), summarized below. The standard error of the reference method (SEL, also called reproducibility) was calculated as the mean of the standard deviations of the difference between the duplicates of six samples of the calibration set that were measured at a three-month interval. The raw NIR spectra were preprocessed using Savitsky-Golay algorithm to compute

smoothed (noise reduction), first derivative (offset and bias removal) spectra. The smoothing window did not eliminate any important feature of the spectra. Accordingly, all the relevant chemical informations were retained for modeling. The NIRM model was built with the following workflow: (1) establishing a NIRM calibration model for target compositions and then optimizing this model; (2) using validation sets to verify the accuracy and repeatability of this model and (3) finally, to improve the accuracy of the prediction, the calibration and validation sets were combined to elaborate the final NIRM model.

The development of the NIRM calibration model linking NIRM data (X) with chemical data (Y) was performed using Partial Least Squares (PLS) regression and a cross-validation procedure (Martens and Naes, 1989; Saeys *et al.*, 2005). The number of latent variables was selected by the software. In PLS the decomposition of X during regression is guided by the variation in the reference data (Y) (*i.e.*, that the variation in X directly correlated with Y is extracted by maximizing the covariance between X and Y). In quantification, Y contains continuous data obtained from a reference method (*e.g.*, N % and C % in this case). For technical details on PLS, see Abdi (2010). A cross-validation with the leave-one-out method was performed by dividing into 2 segments the data matrix, containing 15 or 30 samples, respectively, for the calibration and final NIRM models.

The accuracy of each calibration (for the calibration and final NIRM models) was evaluated based on the coefficients of determination (R²) for predicted *versus* measured compositions in cross-validation and prediction, and the ratio of prediction to deviation (RPD) (Saeys *et al.*, 2005). The RPD showed the ratio between the standard deviation (SD) of data set to standard error of calibration (SEC) or standard error of cross-validation (SEC_{cv}). The SEC, which expresses the accuracy of

NIR results corrected for the mean difference between NIR and reference methods (bias), was calculated by the equation (1) (Sørensen *et al.*, 2007):

SEC =
$$\sqrt{\frac{1}{n-1}\sum(x_i - y_i - bias)^2}$$
 (1)

where $x_i - y_i$ is the difference between results obtained by the NIRM method (x_i) and reference method (y_i) on sample *i*, and *n* is the total number of independent samples in the test. Bias is the difference between the average of results obtained by the NIRM method (x_i) and reference method (y_i) on sample (Sørensen *et al.*, 2007).

In the validation step of the calibration model, the determination coefficient of prediction (R^{2}_{P}), the standard error of prediction (SEP) and the root mean square of prediction errors (RMSE_P) values was used to evaluate the accuracy of the model (Dardenne, 2010). The RMSE_P was calculated from the difference between NIRM and reference results by the following equation (2) (Sørensen *et al.*, 2007):

$$RMSE_P = \sqrt{\frac{1}{n}\sum(x_i - y_i)^2} \quad (2)$$

where xi – yi is the difference between results obtained by the NIRM method (xi) and reference method (yi) on sample i, and n is the total number of independent samples in the test. The residual standard deviation (RSD) was represented the errors after bias and slope correction or the errors along the calculated single regression line (with a loss of two degrees of freedom) (Kuss, 2003).

The R^2 was obtained for the models according to the following equation (3) (Dardenne, 2010):

$$R^{2}=(SD^{2}-SEC^{2})/SD^{2}$$
 (3)

For the validation step of the calibration model, SEC was replaced by SEP.

The accuracy of the predictions for the models was considered as excellent when R² ≥ 0.91 , good when $0.90 \ge R^2 \ge 0.82$, moderately successful when $0.81 \ge R^2 \ge 0.66$, and unsuccessful when R² ≤ 0.65 (Saeys *et al.*, 2005). In this study, five levels of prediction accuracy were considered for the RPD value of the calibration and the final NIRM models. The accuracy of the intermediate NIRM and final NIRM calibration model was considered unreliable for a RPD < 1.5, a RPD between 1.5 and 2.0 allowed to distinguish the high and low values, good for a RPD between 2.0 and 2.5, a value between 2.5 and 3 allowed to an approximate quantitative predictions and excellent for a RPD > 3 (Saeys *et al.*, 2005). The RPD was directly linked to R² (RPD = $1/\sqrt{1 - R^2}$) and the RPD was anyway more discriminant than R² especially when high R² is close to 1 (Dardenne, 2010). The interpretation of the prediction accuracy of different models considered.

3.3. Results and discussion

3.3.1. Spectra description

A total of 2.160 raw spectra were obtained from an acquisitions at 10 different spatial locations on each of the 216 samples. The chemometric evaluation has been based on the spectra average of each sample. Most of the variation between locations and samples was observed in the absorbance from 1.887 to 2.439 nm (5.300 to 4.100 cm⁻¹) range. **Figure 3-2** illustrates the similarities between near-infrared spectra for one of our samples analyzed by NIRM and by classical NIRS instrument in the range between 1.100 to 2.500 nm (9.091 to 4.000 cm⁻¹) with a spectral resolution of 8 cm⁻¹. As mentioned earlier in the study of Yang *et al.* (2011),

the spectrum characteristics obtained by NIRM correspond to those of NIRS. Main of the absorption bands are observed in the 1.660-2.500 nm (6.024 to 4.000 cm⁻¹) range which is mainly related to carbohydrates, lipids and crude protein (Yang *et al.*, 2011).

The main features of the absorption bands of the two spectra were clearly visible on the **Figure 3-2**. No differences in the bands position or in the shape were observed between the spectra acquired NIRS and NIRM technologies. The spectra could be decomposed into 7 main sections from low to high wavelengths (**Figure 3-2**).



Figure 3-2. Comparison between the near-infrared spectra of one of our samples analyzed by NIRM instrument (continuous line; Hyperion, Bruker Optics, Germany) and by NIRS classical instrument (dotted line; XDS, Foss, Denmark) with the attribution of the main infrared bands (A to G). For sake of clarity, spectra were shifted on the Y (Absorbance) axis.

The first one was characteristic of the second overtone of symmetric and antisymmetric C-H stretch vibration (-CH, -CH₂ and -CH₃ groups) absorption (A) from 1.100 to 1.390 nm (9.091 to 7.194 cm⁻¹). These absorption bands are related to the content in carbohydrates, lipids and proteins (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014; Krämer *et al.*, 2015). The second region was characteristic of the first overtone of the O-H vibration bands and the intermolecular H-bridges of water absorption (B) from 1.390 to 1.660 nm (7.194 to 6.024 cm⁻¹).

There was also an overlap with the combination of two stretches and one deformation of C-H bonds producing this broader NIR absorption band, related to the content in carbohydrates and lipids (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014; Krämer *et al.*, 2015).

The third region was characteristic of the first overtone of symmetric and antisymmetric C-H stretch vibration (-CH₂ and -CH₃ groups) absorption (C) at 1.660 and 1.870 nm (6.024 and 5.348 cm⁻¹), respectively. These absorption bands are related to the content in lipids and proteins (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014; Krämer *et al.*, 2015).

The next region includes absorption bands characteristic of the first overtone (D) from 1.870 to 2.015 nm (5.348 to 4.963 cm⁻¹). These absorption bands are mainly related to the content in carbohydrates (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014). The broader absorption around 1.934 nm (5170 cm⁻¹) was also due to an overlap with combinations derived from the vibration of O-H bands characteristic of absorption by water and fundamental vibrations of ester bands (C=O) that occur in the MIR region (Westad *et al.*, 2008; Barbin *et al.*, 2015).

The fifth region was characteristic for the absorption of C-H, N-H and C=O bonds present in carbohydrates, lipids and proteins (E) from 2.015 to 2.230 nm (4.963 to 4.484 cm⁻¹), corresponding to the combination of C-H stretching and bending modes of methyl (-CH₃) and methylene (-CH₂) functional groups (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014; Krämer *et al.*, 2015).

The next region is characteristic of the C-H combination bands for carbohydrates, lipids and proteins absorption (F) from 2.230 to 2.360 nm (4.484 to 4.237 cm⁻¹) (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014; Krämer *et al.*, 2015).

The last region is characteristic of the C-H combination bands for lipids and proteins absorption (G) from 2.360 to 2.500 nm (4.237 to 4.000 cm⁻¹) (Williams, 2001; Baeten and Dardenne, 2002; Westad *et al.*, 2008; Barbin *et al.*, 2014; Krämer *et al.*, 2015).

3.3.2. Reference analysis

The minimum, maximum, mean, and standard deviation (SD) of the nitrogen and carbon content (N and C-value in %) in the calibration and validation sets are shown in **Table 3-2**.

	Calibration set		Validation set		
Units	% N	% C	% N	% C	
Ν	30	30	30	30	
Min	1.03	31.33	1.03	35.44	
Mean	2.09	37.27	1.71	38.79	
Max	3.08	40.00	3.12	41.86	
SD	0.64	1.50	0.60	1.11	

Table 3-2. Characteristics of the calibration and validation sets.

Units: percentage of N or C; N: number of samples; Mean: average; SD: standard deviation; Min: minimum; Max: maximum.

The value range for the nitrogen and carbon content in the calibration and validation sets were similar which means that the calibration and validations sets can be used to establish, test and verify the accuracy of the NIRM model. Reference values of calibration and validation sets were showed in **Supplementary Table S1**.
3.3.3. NIRM calibration and validation

NIRM calibration models were developed for the nitrogen (models 1, 2 and 3) and carbon (model 4) content determination using the 30 samples of the calibration set and the differences between the models are summarized in **Table 3-3**.

In accordance with the recommendations of Dardenne (2010), **Table 3-3** summarizes the characteristics of the models constructed. The calibration step highlights the presence of 3 outliers for the N-value. For the nitrogen content calibration, the best compromise for the number of terms used to derive the calibration was 5 or 3, respectively for models constructed without (*e.g.*, raw data) or with pre-treatments (*e.g.*, smooth and derivative). The low difference between the standard error of calibration (SEC) and the standard error of cross-validation (SEC_{cv}) for the N-content models was indicated a sufficient number of samples for the calibration. In this study, the determination coefficient of calibration (R^2_c) of the first model (model 1) was 0.86 and the SEC_{cv} was 0.31 (**Table 3-4**).

Tahla	3-3	Calibrati	on mod	olc dif	foroncos
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Model	Variable	Pre-treatement	Outlier
1	Ν	None	None
2	Ν	Yes	None
3	Ν	Yes	Yes
4	С	Yes	None

Model: model number constructed (1, 2, 3 and 4); **Variable**: Nitrogen (N) or Carbon (c); **Pre-treatment**: with or without data pre-treatment; **Outliers**: with or without excluded values.

The calibration models with pre-treatments, have R^2_c values were 0.90 and 0.98, respectively good for model 2 and excellent for model 3. The determination coefficient of cross validation (R^2_{cv}) values were closely aligned with the R^2_c values for both calibration models (**Table 3-4**), albeit typically a little weaker than R^2_c . SEC_{cv}

for the nitrogen content determination were 0.27 and 0.14, respectively for model 2 and model 3.

Parameters	N-value	N-value	N-value	C-value
Model	1	2	3	4
Units	% N	% N	% N	% C
SEL – reproducibility	0.05	0.05	0.05	1.19
Ν	30	30	30	30
Outliers	0	0	3	0
Min	1.03	1.03	1.03	31.33
Mean	2.09	2.09	2.07	37.27
Max	3.08	3.08	3.04	40.00
SD	0.64	0.64	0.635	1.50
SEC	0.22	0.20	0.09	1.32
R ² _C	0.86	0.90	0.98	0.20
SEC _{CV}	0.31	0.27	0.14	1.45
R ² _{CV}	0.78	0.81	0.95	0
Reproducibility - between days	0.32	0.32	0.32	3.52
Number of terms	5	3	3	3
RPD _c	2.67	3.16	7.07	0.88
RPD _{cv}	2.13	2.29	4.47	0.97
Segments (CV)	2	2	2	2
WL Range/Resolution (nm)	1111- 2500/2	1111- 2500/2	1111- 2500/2	1111- 2500/2
Pre-treatement(s)	NONE	SG-D1 (15, 2)	SG-D1 (15, 2)	SG-D1 (15, 2)
Reg. Method	PLS	PLS	PLS	PLS

Table 3-4. Characteristic of the NIRM models constructed.

Model: model number constructed on all sample or without outliers; Units : percentage of N or C; SEL – reproducibility: standard error of the reference method; N: number of samples; Outliers: number of values excluded; Min: minimum; Mean: average; Max: maximum; SD: standard deviation for N or C-values of the calibration set; SEC: standard error of calibration; R²_C: determination coefficient of calibration; SEC_{cv}: standard error of cross-validation; R²_{Cv}: determination coefficient of cross-validation; Reproducibility between days: variability of the measurements between days; Number of terms: number of terms in the equation; RPD_c: ratio of prediction to deviation of calibration; RPD_{cv}: ratio of prediction to deviation procedure; WL range/step (nm): range of wavelengths explored and resolution in nm; Pre-treatment(s): data pre-treatment apply on raw spectra; Reg. Method: regression method used.

54

In this study, the ratio of prediction to deviation of calibration (RPD_c) for the nitrogen content calibration were 3.16 and 7.07 respectively for the calibration model 2 and model 3. These results correspond to excellent models (Saeys *et al.*, 2005).

For the carbon content calibration, the best model built has a R^2_c value of 0.20 (model 4) and a RPD_c value of 0.88 (**Table 3-4**).

In accordance with Saeys *et al.* (2005), this results indicate that it was not possible to build a successful calibration. Trial to build a successful calibration has been also done using a databases made on the 60 samples used for the calibration and the validation sets. The best R^2_c value was 0.30 and RPD_c value was 0.84. To conclude, a good model to predict C-value content in tomato leaves powder could not be achieved.



Figure 3-3. (A) Plot of % N in the *Solanum lycopersicum* L. samples analyzed in the calibration stage. Results of the reference values vs NIRM prediction (Model 3) are plotted. NIRM calibration (o) and cross-validation (•) results are displayed. **(B)** Plot of % N in the *Solanum lycopersicum* L. samples analyzed in the validation stage. Results of the reference values vs NIRM prediction (Model 3) are plotted.

Model 3 (pretreatment and outliers exclusion) was selected on the basis of the SEC, R_c^2 and RPD_c values and was tested on the validation set. Figure 3-3.A displays calibration and cross-validation results (the reference values *versus* NIRM predicted values) of Model 3 for determination of N-value in %.

The performance of NIRM model 3 was tested on the 30 independent samples of the validation set (**Table 3-5**). The determination coefficient of prediction (R^{2}_{p}) obtained on the validation set was 0.93 for the nitrogen content determination (**Table 3-5**). This result of the validation step indicates that the accuracy of the predictions of NIRM model 3 was excellent ($R^{2} \ge 0.91$).

Parameters	N-value		
Units	% N		
Ν	30		
Outliers	0		
Min	1.03		
Mean	1.71		
Max	3.12		
SD	0.60		
R ² _P	0.93		
RMSE _P	0.18		
SEP	0.16		
RSD	0.20		
Reproducibility - between days	0.32		
Bias	-0.09		
Intercept	0.06		
Slope	0.91		

Table 3-5. Statistics of the NIRM model 3 validation for the nitrogen determination.

Units : percentage of N; N: number of samples; Outliers: number of values excluded; Min: minimum; Mean: average; Max: maximum; SD: standard deviation for N-values of the validation set; SEC: standard error of calibration; R²_P: determination coefficient of prediction; RMSE_P: root mean square errors of prediction; SEP: standard error of prediction; RSD: the residual standard deviation; Reproducibility between days: variability of the measurements between days; Bias: deviation of the regression line; Intercept: regression constant; Slope: coefficient de regression.

The standard error of prediction (SEP) obtained on the independent validation set was 0.16 for the nitrogen content determination. The SEP of NIRM model is expected to be equal or superior to the standard error of reference method (SEL, also called

reproducibility). In this NIRM model (**Table 3-5**), the SEP value (0.16) was just three times higher than the SEL values (0.05). The SEP value demonstrates the possibility to predict accurately the N content. The root mean square error of prediction (RMSE_P) obtained using Partial Least Square (PLS) after pre-processing optimization was 0.18 for total nitrogen content. **Figure 3-3.B** presents the reference values versus NIRM predicted values obtained for N-content (in %) for the samples of the validation set.

In order to improve the accuracy of the prediction, the data of the calibration and validation sets (60 samples) were combined to elaborate the models 5 and 6, respectively, with and without outliers (**Table 3-5**). A cross-validation procedure was used to evaluate the quality of these models. Four outliers were highlighted for the N-value final set of samples. The narrow gap between SEC and SEC_{cv} for models 5 and 6 indicated that the number of samples included in the study is adequate.

The SEC values obtained for models 5 and 6 were 0.18 and 0.11 respectively, about two and three times higher than the SEL (0.05) of the reference method (**Table 3-6**). The increase in the number of samples achieved by combining the two sets therefore improved the performances of the model (Models 3 and 6 have, respectively, a SEP of 0.16 (**Table 3-4**) and a SEC of 0.11 (**Table 3-6**)). The coefficient of determinations (R^2_c) obtained for models 5 and 6 were 0.91 and 0.97 respectively, indicating that the performances of the two models were excellent. Finally, models 5 and 6 yield RPD_c of 3.33 and 5.77 respectively, which correspond to excellent prediction models. Model 6 (pretreatment and outliers exclusion) was finally selected on the basis of the SEC, R^2c and RPDc values to make predictions. Predictions results of the model 6 were showed in **Supplementary Table S2**.

Parameters	N-value	N-value
Model	5	6
Units	% N	% N
SEL – reproducibility	0.05	0.05
Ν	60	60
Outliers	0	4
Min	1.03	1.03
Mean	1.90	1.86
Max	3.12	3.04
SD	0.64	0.62
SEC	0.18	0.11
R ² c	0.91	0.97
SEC _{CV}	0.22	0.14
R ² _{CV}	0.89	0.95
Reproducibility - between davs	0.32	0.32
Number of terms	3	3
RPDc	3.33	5.77
RPD _{cv}	3.02	4.47
Segments (CV)	2	2
WL Range/Resolution (nm)	1111-2500/2	1111-2500/2
Pre-treatement(s)	SG-D1 (15, 2)	SG-D1 (15, 2)
Reg. Method	PLS	PLS

Table 3-6. Characteristics and statistics of the final NIRM model.

Model: model number constructed on all sample or without outliers; **Units** : percentage of N; **SEL** – reproducibility: standard error of the reference method; N: number of samples; **Outliers**: number of values excluded; **Min**: minimum; **Mean**: average; **Max**: maximum; **SD**: standard deviation for N-values of the calibration and validation sets combined to elaborate the final model; **SEC**: standard error of calibration; **R**²c: determination coefficient of calibration; **SEC**_{cv}: standard error of cross-validation; **R**²cc: determination coefficient of cross-validation; **R**²cc: attached error of the measurements between days; **Number of terms**: number of terms in the equation; **RPD**c: ratio of prediction to deviation of calibration; **RPD**cv: ratio of prediction to deviation of cross-validation procedure; **WL range/step (nm)**: range of wavelengths explored and resolution in nm; **Pre-treatment(s)**: data pre-treatment apply on raw spectra; **Reg. Method**: regression method used.

3.4. Conclusions

Our study demonstrates the feasibility of accurately estimating the N content of very small tomato leaf samples using the NIRM technique. The main benefits of this technique compare to conventional methods (*e.g.* the Kjeldahl method, the Dumas method and NIRS) lays essentially in the simple sample preparation procedure, involve small analytical costs and times and in the small amount of tissue that is required. This innovation should ease (i) the establishment of N profiling among different organs of the same plant, (ii) the dynamic monitoring of N content in time for a given plant and (iii) the development of high throughput methods of N quantification in studies involving large numbers of genotypes. Conditional on further validation, the method may also proved very useful for small plants such as *Arabidopsis thaliana* where large amounts of plant material often requires the pooling of several plants. In a N profiling strategy, the methodology may also be used to produce local observations within a leaf, especially in the study of defense mechanisms against leaf diseases.

One may expect the NIRM methodology to be used for predicting other physical, chemical and biological properties and for embracing different aspects of the plant phenotype or phenotypic responses to various factors. The potential of the NIRM method to detect plant stress due to abiotic factors (*e.g.*, nutrients, salinity) and to determine the chemical and physical properties in several plants tissues and samples (*e.g.*, whole plants, fruits, grains, leaves) has been demonstrated already (Cozzolino, 2009; Dale *et al.*, 2011; Tuccio *et al.*, 2011, Alander *et al.*, 2013; López *et al.*, 2013; Barbin *et al.*, 2014; Pojić *et al.*, 2015; Wang *et al.*, 2014; Xin *et al.*, 2015). The ongoing technical improvements of NIRM will offer new perspectives and solutions for a fast,

reliable, environmentally-friendly testing and simultaneously quantification of physical, chemical and biological plant properties.

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Authors' contributions

G. Lequeue performed experiments, analyzed data and drafted the manuscript. X. Draye and V. Baeten helped to design the study and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no competing financial interests. The views and opinions expressed in this article are purely those of the writers and may not in any circumstances be regarded as stating an official position of the European Commission.

60

Supporting information

Supplementary Table S1 and S2 are available at http://www.nature.com/articleassets/npg/srep/2016/160916/srep33183/extref/srep33183-s1.pdf (file format: PDF).

Chapter 4

Assessment of diagnostic optical tools for the evaluation of N content in tomato (*Solanum lycopersicum L*.) cuttings.

As an alternative to the methods described in chapter 3, we have investigated direct and indirect non-invasive measurements of chlorophyll content and fluorescence as real-time proxies of nitrogen content and deficiency. These methods were evaluated with tomato (*Solanum lycopersicum* L.) plants grown at different nitrogen supplies. Available physiological and morphological variables observed in chapter 3 and chapter 4 have then been used to identify possible combinations of variables which allow to discriminate the different temporal responses to nitrogen supply over time. This study has led to the selection of a realistic method for high throughput phenotyping in chapter 5.

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63

Abstract

Sustainable and precision nitrogen (N) management strategies aim at fitting N fertilizer inputs to the spatial and temporal variability of N supply from the soil and demand by the crop. The aim of this study was to compare existing N sensor technologies to estimate the N content of tomato plants (*Solanum lycopersicum* L.). We used a classical chlorophyll meter (CCM-200) and two different types of active fluorimeters (PEA fluorimeter and Mutiplex330). The experiment was conducted in a greenhouse with potted tomato plants that were subjected to six levels of nitrogen supply (*i.e.*, 0.41, 0.81, 1.63, 3.25, 6.50 and 13 mmol.l⁻¹). Our results indicate that Multiplex parameters were highly sensitive to applied N and could be used to estimate N supply from plant optical properties.

4.1. Introduction

Nitrogen (N) is a major determinant of biomass production. N deficiency, even temporary, usually leads to significant yield reductions, while N inputs in excess to the needs of crops lead to a reduction in nitrogen use efficiency (Tremblay *et al*, 2012; Goffart *et al.*, 2013; Ben Abdallah *et al*, 2016). Recent precision N management strategies aim to optimize N fertilizer inputs by considering the spatial and temporal variability of N demand by the crop and N supply from the soil, in order to improve N use efficiency and protect the environment (Miao *et al.*, 2007; Miao *et al.*, 2011; Diacono *et al.*, 2013; Ben Abdallah *et al*, 2016).

Precision N management rely on our ability to monitor crop nitrogen as they are based on the consideration that the crop itself is a suitable indicator of its N requirements and satisfaction. The crop biomass, for example, is often considered as an indicator integrating the effects of the environmental conditions that determine plant growth, including N supply (Tremblay *et al*, 2012; Goffart *et al.*, 2013; Ben Abdallah *et al*, 2016).

To be usable in practice, rapid assessment tools must meet four main conditions, base on Gianquinto *et al.*, 2004; Tremblay *et al.*, 2012; Goffart *et al.*, 2013; Ben Abdallah *et al*, 2016:

- the measure must be fair and accurate, *i.e.* the values should be close to the actual value and be repeatable;
- the measurement must be sensitive, *i.e.* the early detection of nitrogen deficiency should be possible to allow the application of an adequate complementary dose;

- the measurement should be specific, *i.e.* it should ideally be exclusively connected with the nitrogen concentration of the plant without interference with other external factors;
- the method should be simple, inexpensive and fast to allow its wide use by farmers or consultants.

Consequently, there has been increasing interest in using proximal and remote sensing technologies to estimate plant N status, at the scales of the tissue, leaf, plant or canopy (Houlès *et al.*, 2007; Mistele and Schmidhalter, 2008; Ziadi *et al.*, 2008; Cao *et al.*, 2012; Diacono *et al.*, 2013; Cao *et al.*, 2015; Ben Abdallah *et al.*, 2016).

Several noninvasive methods exist to determine the chlorophyll concentration (Booij et al., 2000; Houlès et al., 2007; Mistele and Schmidhalter, 2008; Ziadi et al., 2008; Gianquinto et al., 2011; Tremblay et al., 2011; Cao et al., 2012; Tremblay et al., 2012; Diacono et al., 2013; Cao et al., 2015; Ben Abdallah et al, 2016). The most commonly used rely on a chlorophyll meter which yields chlorophyll concentration in individual leaves (Debaeke et al., 2006; Prost and Jeuffroy, 2007; Ziadi et al., 2008; Cao et al., 2012; Cao et al., 2015; Ben Abdallah et al, 2016). A complete review of the potential and the use of chlorophyll, the scientific model, SPAD-502 (Minolta, Osaka, Japan) or its commercial equivalent, the Hydro N-test (NST - Yara, Oslo, Norway) to estimate the nitrogen status of potato (Solanum tuberosum L.) was carried out by Gianquinto et al. (2004). The application of this method was successfully adopted on several crops, such as tomato (Solanum lycopersicum) (Ulissi et al., 2001; Gianquinto et al., 2006), cantaloupe (Cucumis melo var. cantalupensis) (Orsini et al., 2009; Gianquinto et al., 2011), and friarielli (Brassica rapa ssp. Sylvestris) (De Pascale et al., 2008). The main advantages of this method are the low variability between independent values and the ease of use (Gianquito et al., 2004; Padilla et al., 2014; Ben Abdallah et al, 2016). The measurements, however, achieve a weak discrimination between increasing doses of nitrogen, except for situations with little or no fertilized (Gianquito *et al.*, 2004; Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016).

Methods based on **chlorophyll fluorescence** are also used for estimating the nitrogen status of individual leaves (Tremblay 2004; Ben Abdallah *et al*, 2016). These methods are based on induced chlorophyll fluorescence in combination with the absorption of UV rays by polyphenolic compounds in the epidermis leaf (Campbell *et al.*, 2007; Tremblay *et al*, 2012; Ben Abdallah *et al*, 2016). These methods have a higher sensitivity to assess the nitrogen status of the culture since the variation of the concentration of phenols (flavonols) in relation to the nitrogen status can be detected before the chlorophyll content and leaf area index are modified (Cartelat *et al.*, 2005). The Dualex and Multiplex (Force-A, Université Paris-Sud, Orsay, Paris, France), which are based on these principles, have been evaluated to estimate the N status of corn (Tremblay *et al.*, 2007; Zhang *et al.*, 2012; Longchamps and Khosla, 2014).

Indices combining the concentrations of flavonols and chlorophyll have been proposed as reliable proxies of plant N status (Cartelat *et al.*, 2005; Tremblay *et al.*, 2007; ; Cerovic *et al.*, 2012; Agati *et al.*, 2013; Longchamps and Khosla, 2014; Ben Abdallah *et al*, 2016). They improve the early discrimination between N treatments and are stable over time (Cartelat *et al.*, 2005; Tremblay *et al.*, 2007; Cerovic *et al.*, 2012; Agati *et al.*, 2005; Tremblay *et al.*, 2007; Cerovic *et al.*, 2012; Agati *et al.*, 2005; Tremblay *et al.*, 2007; Cerovic *et al.*, 2012; Agati *et al.*, 2013; Longchamps and Khosla, 2014; Ben Abdallah *et al.*, 2016).

The aim of this study was to compare existing N sensor technologies to detect N variation on derooted tomato (*Solanum lycopersicum* L.) growing under nitrogen deficiency. To this end, plant growth, biomass and also photosynthesis were analyzed using a chlorophyll meter and two different types of fluorimeters. These

methods were compared with N measurements obtained with a reference analysis methods (NIRM, see Chapter 3).

4.2. Materials and methods

4.2.1. Plant material and growth conditions

The study was conducted in Louvain-la-Neuve, from 23 July 2013 to 12 September 2013. Seeds of the tomato (*Solanum lycopersicum* L.) variety Ailsa Craig were surface-sterilized by soaking in a 5 % (v/v) sodium hypochlorite solution for 15 min and rinsed three times with deionized water. They were germinated in a loam substrate incubated in a growth chamber (24 °C / 22 °C; 80 % RH; 16 h photoperiod; 150 μ mol.m⁻².s⁻¹ PAR). Ten days after sowing, seedlings were washed with deionized water and transferred individually in 1.45 L pots filled with a mix of perlite and vermiculite (50/50).

Seven days later, the plantlets were acclimated in a greenhouse during seven days. A data-logger (TinyTag Ultra, model TGU-1500, INTAB Benelux, Netherlands) was used to record climate data during the experiment (T_{mean} 26.5/18.2 °C day/night, (max. 34.8/27.9 °C day/night, min. 13.1/12.8 °C day/night) and RH_{mean} 52.8/69.0 % day/night (max. 93.5/96.4 % day/night, min. 27.8/41.6 % day/night). The photoperiod was set at 16 h and the solar radiation was supplemented with Philips HPLR lamps (400 W) providing 40 µmol m⁻² s⁻¹ at the canopy level. During these periods, plants were watered three times per week using a modified Hoagland solution with a nitrogen concentration of 13 mmol.l⁻¹ (Table 4-1).

After acclimation, plants were organized in six groups of 12 plants assigned to one of six nitrogen treatments (13.0; 6.50; 3.25; 1.63; 0.81; 0.41 mmol.l⁻¹) (**Table 4-1**).

Chapter 4

They were watered three times per week, throughout the experiment, with a volume of 100 ml solution from the top of the pot.

Table 4-1. The principal chemical compounds (in mmol.l⁻¹) with the different electrical conductivity (EC in dS/M) and pH used in nitrogen solutions (1.3; 6.50; 3.25; 1.63; 0.81; 0.41 mmol.l⁻¹) achieve from a modified Hoagland solution.

Elements	Solutions					
[Total nitrogen] (in mmol.l ⁻¹)	13	6.50	3.25	1.63	0.81	0.41
NO3⁻ (in mmol.l ⁻¹)	12.00	6.00	3.00	1.50	0.75	0.38
NH4+ (in mmol.l ⁻¹)	1.00	0.50	0.25	0.13	0.06	0.03
SO4 ²⁻ (in mmol.l ⁻¹)	1.00	3.75	5.13	5.82	6.16	6.33
H2PO4 ⁻ (in mmol.l ⁻¹)	2.01					
K ⁺ (in mmol.l ⁻¹)	6.01					
Ca ²⁺ (in mmol.l ⁻¹)	3.50					
Mg ²⁺ (in mmol.l ⁻¹)	1.00					
рН	5.6					
EC (dS/m)	1.60	1.51	1.53	1.51	1.51	1.50

Three replications of the experiment were conducted in parallel, yielding a total of 216 samples.

4.2.2. Biomass and leaf area measurements

Every week following the onset of the treatments, three plants from each treatment were harvested. Their leaf area was determined using an imaging method. The shoot and root parts were also weighed and dry matter content determined after ovendrying at 65 °C until constant weight.

4.2.3. Optical sensor measurements

Fluorescence emission was assessed between 9:00 and 12:00 am using a plant efficiency analyzer (PEA fluorimeter, Hansatech Instruments Ltd, Norfolk, United Kingdom). Leaf discs were dark-adapted during 30 min dark adaptation with a leafclip, delimiting a leaf surface area of 0.50 cm² and exposed to a saturation pulse of high light intensity (2250 µmol m-2 s-1) for five seconds. The fluorescence variables Fo, Fm, Fv and Fv/Fm were determined.

Indirect measurements of leaf chlorophyll content were made with a hand-held leafclip (with a measure surface of 0.71 cm²) chlorophyll meter (CCM-200, Opti-Sciences Inc., New Hampshire, USA).

Fluorescence measurements emitted by plant fluorescent pigments (fluorophores) after excitation were carried out using a hand-held multi-parameter fluorescence sensor (Multiplex Research[™], FORCE-A, Orsay, France). The instrument generates fluorescence using four excitation channels (UV about 375 nm, blue about 450 nm, green about 530 nm and red about 630 nm) and records the fluorescence emitted by plant material in the following bands: blue-green (447 nm) when blue excitation is not used or yellow (590 nm) when bleu excitation is used; red (665 nm) and farred (735 nm). More of 20 Multiplex parameters are computed for each measurement (Table 4-2). The Multiplex can be used to estimate the content of various compounds such as anthocyanin (epidermal visible absorbance by Fluorescence Excitation Ratio (FER) method), flavonol (epidermal UV absorbance by FER method) and chlorophyll, as well as several other fluorescence parameters (such as ANTH-RG, FER_RG, FLAV, SFR-G, NBI-R, FERARI) that have been identified as indicative of plant physiological status. The Multiplex measures a leaf surface area of 50 cm². More details about the sensor can be found in Force A (2017), Ben Ghozlen et al. (2010) and Tremblay et al. (2012).

Chapter 4

Multiplex Description Target Excitation Formula parameters BGF-UV Yellow Fluorescence UV 1 1 RF-UV Red Fluorescence / UV / FRF-UV Far-Red Fluorescence / UV / BGF-B Yellow Fluorescence / Blue / RF-B Red Fluorescence 1 Blue 1 1 FRF-B Far-Red Fluorescence 1 Blue 1 BGF-G Reflected Yellow-Green ligth Green / RF-G **Red Fluorescence** 1 Green 1 FRF-G Far-Red Fluorescence 1 / Green BGF-R Reflected Yellow-Red ligth 1 Red / RF-R Red Fluorescence 1 Red / FRF-R Far-Red Fluorescence 1 Red 1 Fluorescence Excitation Red and 1 FFR-RUV / Ratio UV Fluorescence Excitation Red and FER-RG / / Ratio Green Fluorescence Excitation Red and FER-RB 1 / Ratio blue Chlorophyll Index (Simple SFR-G Chlorophyll Green FRF-G/RF-G Fluorescence Ratio) Chlorophyll Index (Simple SFR-R Chlorophyll FRF-R/RF-R Red Fluorescence Ratio) Blue to Red Fluorescence Complex – grape BGF-UV/FRF-UV BRR-FRF UV Ratio maturation Red and Log (FER-RUV) = FLAV Flavonols Index Flavonols Log(FRF-R/FRF-UV) UV Red and Log (FER-RG) = ANTH-RG Anthocyanins Content Index Anthocyanins Log(FRF-R/FRF-G) Green Red and Log (FER-RB) = ANTH-RB Anthocyanins Content Index Anthocvanins blue Log(FRF-R/FRF-B) Epidermal phenolics and UV and FRF-UV/RF-G NBI-G Nitrogen Balance Index chlorophyll Green Epidermal phenolics and UV and NBI-R Nitrogen Balance Index FRF-UV/RF-R chlorophyll Red Anthocyanin Relative FERARI Anthocyanins Red - log(FRF_R) Index

Table 4-2. Characteristic of the physical and the physiology (grey background) parameters directly related to the physiological status of plants provided by the Multiplex adapted from Zhang *et al.*, 2012.

Measurements with these three devices started on the same day as the nitrogen treatments. CCM-200 and Multiplex measurements were made every day, while measurements with the PEA fluorimeter were made weekly, until the end of the experiment. Measurements were always made at the same period (7:00 to 14:00), firstly with the CCM-200, then with the Multiplex and finally with the PEA fluorimeter. Measurements with the CCM-200 and with the PEA fluorimeter were made on the distal part of the adaxial side of the third leaf (fully expanded and well lit), half-way between the margin and the mid-rib of the leaf. The Multiplex was positioned for measurement at a 10 cm distance above the plant.

In addition, chlorophyll (Chl a and Chl b) and total carotenoid (xantophylls + β carotene) concentrations were quantified using a spectrophotometric method modified from Lichtenthaler (1987). The third fully expanded leaves of each plant were chosen, placed between layers of ice in a thermal insulated box. Leaf samples (0.1 g fresh weigh or 3.0 cm2) were ground in an aqueous acetone solution (80% v/v) containing sea sand (0.5% w/v). Samples were then centrifuged at 3000 x g for 10 min at 4°C. The supernatant was used for determining absorbance at 470, 646.8 and 663.2 nm to estimate carotenoid and chlorophyll concentrations.

4.2.4. NIR microscopy approach

A total of 216 shoot dry samples were crushed with a sample mill (CT 193 Cyclotec[™], Foss, Hillerød, Denmark) to obtain a powder (within a dry matter weight interval between 0.01 and 5.2 g). NIR analysis was performed using an automated Fourier Transform-IR imaging Microscope (Hyperion 3000, Bruker Optics, Ettlingen, Germany) as described by Lequeue *et al.* (2016) as a reference method. Data were recorded in the range from 9.000 to 4.000 cm⁻¹ with a spectral resolution of 8 cm⁻¹. All spectra were collected with 32 co-added scans. Vibrational spectroscopy was

performed directly on crushed shoot powder. For each sample, 10 spectra were collected at different spatial location of the samples spread on an aluminum plate with 96 wells containing the sample distributed based upon the dry weight available. The subsequent chemometric evaluation has exclusively been based on the average spectrum of each sample..

4.2.5. Statistical analysis

Data were analyzed by means of variance analyses and Student's t-test (P = 0.05) with the statistical software package SAS v.9.4 (SAS Institute Inc., 2013). Figures were also plotted using SAS v.9.4. Time was expressed in thermal time using a base temperature of 12°C.

4.3. Results and discussion

4.3.1. Plant growth

The evolution of leaf number and plant weight is plotted in **Figure 4-1** and the statistical results on all plant growth data are summarized in **Table 4-3**. The effect of plant age, nitrogen input and their interaction were highly significant for most plant growth variables. The nitrogen input had a progressive effect on the number of leaves, but had a rather discrete effect on plant weight, with the 6.5 mM concentration showing an intermediate effect between the control and the other treatments.



Figure 4-1. Number of leaves (a), plant dry weight (b), shoot dry weight (c) and root dry weight (d) as a function of thermal units. Confidence limits ($\alpha = 0.05$) are shown in light gray on the 13 mM treatment. The significance level (* or **) of the treatment effect for each thermal time is indicated at the bottom of the graph.

With the exception of chlorophyll b, the response to N tended to be linearly related to the N input. The effect of thermal time was significant for the three variables, but the N treatment and its interaction with thermal time were only significant for chlorophyll b, due to large residual variances (**Table 4-4**).

In line with this large variance, the temporal evolution of the chlorophyll index (CCM-200) revealed significant differences already at the onset of treatments (**Figure 4-2d**). These differences remained until 100 °C.days and did not correlate with the nitrogen input. After 120 °C.days, the treatments started to differentiate and displayed a correlation with the nitrogen input. The treatment and time effects and their interaction were highly significant (**Table 4-4**).

Variable	Source of variation	Df	F-Value	P-Value
Nb_Leaf	Trt	5	2.99	0.01
Nb_Leaf	Date	5	308.61	<0.001
Nb_Leaf	Trt*Date	25	8.59	<0.001
SFW	Trt	5	72.37	<0.001
SFW	Date	3	554.42	<0.001
SFW	Trt*Date	15	31.55	<0.001
SDW	Trt	5	26.00	<0.001
SDW	Date	3	647.28	<0.001
SDW	Trt*Date	15	13.36	<0.001
SWC	Trt	5	8.51	<0.001
SWC	Date	3	364.25	<0.001
SWC	Trt*Date	15	2.26	0.006
RFW	Trt	5	35.20	<0.001
RFW	Date	3	399.02	<0.001
RFW	Trt*Date	15	15.01	<0.001
RDW	Trt	5	51.15	<0.001
RDW	Date	3	414.77	<0.001
RDW	Trt*Date	15	23.68	<0.001
RWC	Trt	5	1.50	0.193
RWC	Date	3	40.83	<0.001
RWC	Trt*Date	15	3.11	<0.001
FW	Trt	5	65.64	<0.001
FW	Date	3	580.82	<0.001
FW	Trt*Date	15	27.87	<0.001
DW	Trt	5	53.82	<0.001
DW	Date	3	767.89	<0.001
DW	Trt*Date	15	25.28	<0.001
R_S	Trt	5	0.70	0.623
R_S	Date	3	3.57	0.015
R_S	Trt*Date	15	1.55	0.091
LA_cm ²	Trt	5	60.53	<0.001
LA_cm ²	Date	3	388.38	<0.001
LA_cm ²	Trt*Date	15	28.84	<0.001
SLA	Trt	5	1.08	0.375
SLA	Date	3	18.23	<0.001
SLA	Trt*Date	15	1.06	0.397
LAR	Trt	5	0.92	0.471
LAR	Date	3	25.52	<0.001
LAR	Trt*Date	15	0.65	0.833

 Table 4-3.
 Variance analyses of growth-related variables.

Df: Degree of freedom model; Nb_leaf: leaves number; SFW: Shoot fresh weight (in g); SDW: Shoot dry weight (in g); SWC: Shoot water content (in %); RFW: Root fresh weight (in g); RDW: Root dry weight (in g); RWC: Root water content (in %); FW: Plant fresh weight (in g); DW: Plant dry weight (in g); R_S: Root/shoot; LA_cm²: Leaf Area (in cm²); SLA: Specific leaf area (in cm².g-1); LAR: Leaf area ratio (in cm².g-1).

A few tests were not significant: (i) nitrogen input affected the temporal evolution of root water content, but did not affect the average root water content; (ii) the root:shoot ratio was not affected by plant age nor by nitrogen input; (iii) specific leaf area and leaf area ratio were only affected by plant age.

4.3.2. Photosynthetic pigments concentration, chlorophyll index and nitrogen content

Low nitrogen inputs seemed to decrease the concentration of photosynthetic pigments with time, in different ways (**Figure 4-2a-c**). The N effect was expressed as early as 7 days after the onset of treatments for the carotenoids, progressively between 7 and 21 days for chlorophyll a and only after 14 days for chlorophyll b. With the exception of chlorophyll b, the response to N tended to be linearly related to the N input. The effect of thermal time was significant for the three variables, but the N treatment and its interaction with thermal time were only significant for chlorophyll b, due to large residual variances (**Table 4-4**).

In line with this large variance, the temporal evolution of the chlorophyll index (CCM-200) revealed significant differences already at the onset of treatments (**Figure 4-2d**). These differences remained until 100 °C.days and did not correlate with the nitrogen input. After 120 °C.days, the treatments started to differentiate and displayed a correlation with the nitrogen input. The treatment and time effects and their interaction were highly significant (**Table 4-4**).



Figure 4-2. (**a-c**) Effect of the nitrogen input on the concentration (in mg per g fresh matter, relative to the 13 mM treatment) of chlorophyll a (**a**), chlorophyll b (**b**) and carotenoids (**c**) measured at harvest (three time points). (**d-e**) Temporal evolution of the chlorophyll index (**d**) by CCM-200 and of the predicted nitrogen value (in %) by NIRM (**e**) for the six nitrogen inputs. Confidence limits (α = 0.05) are shown in light gray on the 13 mM treatment. The significance level (* or **) of the treatment effect for each thermal time is indicated at the bottom of the graph.

Variable	Source of variation	Df	F-Value	P-Value
Ca_FM	Trt	5	1.59	0.17
Ca_FM	Date	3	27.62	<0.001
Ca_FM	Trt*Date	15	1.29	0.21
Cb_FM	Trt	5	2.57	0.03
Cb_FM	Date	3	16.19	<0.001
Cb_FM	Trt*Date	15	2.57	<0.001
Ccarot_FM	Trt	5	1.03	0.40
Ccarot_FM	Date	3	23.85	<0.001
Ccarot_FM	Trt*Date	15	0.86	0.61
ССМ	Trt	5	3.15	0.008
ССМ	Date	21	104.44	<0.001
ССМ	Trt*Date	105	4.85	<0.001
N_NIRM	Trt	5	36.72	<0.001
N_NIRM	Date	3	182.98	<0.001
N_NIRM	Trt*Date	15	5.67	<0.001

Table 4-4. Variance analyses of photosynthetic pigments concentration, chlorophyll index andNIRM prediction.

Df: Degree of freedom; Ca_FM: Concentration in chlorophyll a; Cb_FM: Concentration in chlorophyll b; Ccarot_FM: Concentration in carotenoids; CCM: chlorophyll index (CCM-200); N_NIRM: predicted nitrogen concentration by NIRM.

The nitrogen concentrations estimated by NIRM were consistent with the CCM values (Figure 4-2e), with significant effects of nitrogen input, time and their interaction. As for CCM, differences between treatments did not correlate with N input until 100 °C.days. The N dependency appeared progressively thereafter, but it took 200 °C.days before clear differences related to N input were visible, showing three groups (0,41-0,81 / 1,63 - 3,25 - 6,50 / 13 mM). A dilution effect of the N concentration over time was clearly observed in all treatments, compared to the 13 mM input which kept the same N concentration throughout the experiment.

4.3.3. Fluorimeter

Low N input decreased the Fo, Fm and Fv an increased the Fv/Fm ratio (**Figure 4-3**). The comparison of the different time points reveals, however, that the N effect was less pronounced at 6 days, most pronounced at 13 days and intermediate at 20 days.



Figure 4-3. Effect of the nitrogen input (mM) on the initial fluorescence (Fo) (**a**), variable fluorescence (Fv) (**b**), maximum fluorescence (Fm) (**c**) and variable/maximum fluorescence (Fm/Fv) ratio (**d**).

This indicates that N deprivation was affecting photosynthesis during the two weeks following the onset of the treatment but that an adaptation occurred which partially reversed the effect of low N. Interestingly, such adaptation was not observed for the

concentration of photosynthetic pigments nor for the nitrogen content. The Fv variable did not display much differences between the different time points and only responded to the N level. The interaction was, therefore, not significant (**Table 4-5**). The Fv/Fm ratio, on the other hand, displayed the most quantitative relationship with the nitrogen dose, except at 6 days after transfer.

Variable	Source of variation	Df	F-Value	P-Value
Fo	Trt	5	18.62	<0.001
Fo	Date	2	9.68	<0.001
Fo	Trt*Date	10	1.94	0.04
Fv	Trt	5	22.65	<0.001
Fv	Date	2	4.98	0.007
Fv	Trt*Date	10	1.06	0.39
Fm	Trt	5	24.20	<0.001
Fm	Date	2	13.32	<0.001
Fm	Trt*Date	10	2.05	0.03
Fv_Fm	Trt	5	16.14	<0.001
Fv_Fm	Date	2	34.88	<0.001
Fv_Fm	Trt*Date	10	2.50	0.007

Tableau 4-5. Variance analysis of fluorescence variables.

Df: Degree of freedom model; Fo: Initial fluorescence; Fv: variable fluorescence; Fm: maximum fluorescence; Fv_Fm: variable/maximum fluorescence ratio.

4.3.4. Multiplex

For each plant and date, the Multiplex device generated 20 variables derived from leaf fluorescence in different wavelengths after excitation by different wavelengths. In order to derive a subset of highly informative variables, we performed principal component analysis which revealed that the 20 variables could be clustered in 8 groups according to their PCA scores on the first three components. Using a backward elimination strategy, we selected a subset of 8 variables (one for each group) which accounted for most of the variability: FERARI, ANTH_RG, FLAV, YF_R,

RF_R, FRF_R, SFR_R and NBI_R. The temporal evolution of these variables for the six N inputs is plotted in **Figure 4-4a-h**. Their power to discriminate different N inputs was rather different, with FLAV and ANTH_RG detecting differences at 70 °C.days already, while FERARI and FRF_R detected differences only at the last measurement. A posteriori, a different selection might have been performed, based on the discrimination of the N inputs.

The discrimination among N treatments using only green-induced fluorescence (*i.e.*, NBI_G) was generally lower than that based on the red-induced fluorescence (data not shown). The characteristics of chlorophyll and carotenoids that absorb mainly blue and red light while they reflect a significant fraction of green light may explain this lower discrimination power of green-induced fluorescence (Seely, 1977; Longchamps and Khosla, 2014). Absorbed green light penetrates deeper in the mesophyll tissues (Buschmann, 2007; Longchamps and Khosla, 2014) where red fluorescence emitted by chlorophyll has more chances to be re-absorbed by surrounding cells. Therefore, the red fluorescence induced by green light (530 nm) is normally lower than the red fluorescence induced by red light (630 nm) (Vogelmann and Evans, 2002; Longchamps and Khosla, 2014). For these reasons and in accordance with the results obtained in Longchamps and Khosla (2014), the red induced fluorescence seemed to provide a better detection power for N variability.

In the PCA performed with the selection of variables, the first and second principal components accounted for 42 and 29.2%, respectively, of the total variance and the eight variables spreaded nicely around the correlation circle (**Figure 4-5a**). The graph on **Figure 4-5b** shows, for each N input level, the temporal trajectory of the sampled plants.



Figure 4-4. (a-h) Temporal evolution of the eight Multiplex variables selected for further analysis. Confidence limits ($\alpha = 0.05$) are shown in light gray on the 13 mM treatment. The significance level (* or **) of the treatment effect for each thermal time is indicated at the bottom of the graph.

Lower N inputs indicated clearly a two-phase trajectory, going first towards the topright quadrant (YF_R, RF_R, FRF_R) then towards the top left quadrant (ANTH_RG, FERARI), while higher N inputs tended to remain in the bottom centered area of the graph (NBI_R).



Figure 4-5. (a) Scores of the eight variables in the first two principal components. **(b)** Trajectories of the different nitrogen dose in the PC1-PC2 plane. Each point is a thermal time. The six trajectories start in the same area of the graph because all plants were equally supplied with 13mM N until the onset of the treatments. This area is marked by a circle (the same on all graphs).

Two reasons may explain the gain of induced fluorescence over active reflectance sensing (*e.g.* CCM). The first reason is that reflectance provides information on the chlorophyll content only, whereas induced fluorescence yields information on two leaf pigments, both phenolics and chlorophyll, for which the concentrations are influenced by the N status of the plant (Mercure *et al.*, 2004; Cartelat *et al.*, 2005; Solari *et al.*, 2008; Longchamps and Khosla, 2014). The second reason is that phenolic compounds are present in younger leaves where a change in their concentration may be detected through higher UV absorption (Crankshaw and Langenheim, 1981; Samson *et al.*, 2000; Longchamps and Khosla, 2014). This is a benefit compared to

canopy reflectance, because nitrogen is easily mobilized in the plant and upper leaves are normally the last organs of the plant where N-deficiency or chlorosis is appearing (Kitchen *et al.*, 2010; Longchamps and Khosla, 2014).

4.3.5. Principal component analysis

A global multivariate analysis including all variables discussed above was carried out in order to identify possible combinations of variables which discriminate the different temporal responses to the N input. This analysis was carried out using only the observation times where all variables had been recorded (6/7, 13/14 and 20/21 days). The first, second and third principal components accounted for 52, 26.2 and 10.1%, respectively, of the total variance (**Figure 5**).

An interesting output of this analysis is that the set of variables provided by the Multiplex device provide a good coverage of all other variables around the correlation circle. This is less the case for NPQ, RWC and Fv/Fm variables that are distant to any Multiplex variables. However, these variables appear opposite to YF_R and SFR_R and are therefore likely to correlate with the opposite of these Multiplex variables.

The representation of the trajectories of the different N treatments indicates a clear separation of the three components. The first component reveals the temporal evolution of plants under low N (right-to-left direction), while the second component reveals the temporal evolution of plants under high nitrogen (bottom-to-top direction). Interestingly, the third component reveals a transient response to low N (bottom-to-top) that is reversed between 13 and 21 days and which corresponds to the evolution of Fv/Fm as reported above.



Figure 4-6. Graphical representation of a principal component analysis performed on the complete set of variables analyzed above. **(a,c)** scores of the variables on the first three components. **(b,d)** projection of the trajectories of the six treatments (over three dates) in the planes of the first three components.

4.3.6. Prediction of N content

Different methods were attempted to evaluate the possibility of predicting nitrogen content using the tested optical methods.

In a first step, seven Multiplex variables were selected from a visual analysis of the univariate relations between nitrogen value and each available variable. The selected variables were RF-UV, NBI-R and G, FRF-UV, FLAV, FER-RUV, and BRR-FRF. Multivariate linear regressions on each experience yielded coefficients of determination of 0.76, 0.79 and 0.56, respectively for the three experiences. The coefficients of determination were only slightly improved by using second order polynomial regression (0.82, 0.79 and 0.57).

In a second step, and in order to build a model in a less subjective manner, we used two methods of model selection. The selected variables were on plant growth (leaves number, shoot fresh and dry weight, shoot water content, root fresh and dry weight, root water content, plant fresh weight, plant dry weight, root/shoot, leaf Area, specific leaf area, leaf area ratio), photosynthetic pigments concentration (chlorophyll a, b and in carotenoids), CCM-200 (chlorophyll index), NIRM prediction (N content), PEA fluorimeter (initial fluorescence, variable fluorescence, maximum fluorescence, variable/maximum fluorescence ratio) and Multiplex (RF-UV, NBI-R and G, FRF-UV, Flav, FER-RUV, and BRR-FRF). The first method was a step wise regression, in which variables are added (or removed) sequentially until the maximum R² is achieved. The selected model comprised two variables and yielded an R² of 0.82. The second model selection method was a backward elimination, which starts with all available variables and sequentially remove variables until no further improvement of a criterion is possible. Different criterions were used, and yielded models with 19, 20 or 21 variables and a R² between 0.88 and 0.94. However,

a unique best model was difficult to determine, probably due to the redundancy between the explanatory variables and the non-linearity in the response to nitrogen.

4.3.7. Conclusion

The results of our study suggest that the Multiplex equipment has a higher potential to estimate the nitrogen content of tomato plants than the classical chlorophyll meter and fluorescence parameters. The added value of induced fluorescence as measured by the Multiplex is likely due to the sensitivity of the method to both phenolic pigments and chlorophyll (Mercure *et al.*, 2004; Cartelat *et al.*, 2005; Solari *et al.*, 2008; Longchamps and Khosla, 2014). In addition, a change in phenolic compounds in response to nitrogen supply seems to be detectable in the young leaves in the top of the canopy (Crankshaw and Langenheim, 1981; Samson *et al.*, 2000; Longchamps and Khosla, 2014), unlike changes in reflectance due to chlorosis that are first detected on old leaves deep in the canopy (Kitchen *et al.*, 2010; Longchamps and Khosla, 2014). The fact that the Multiplex records information on a much larger leaf surface (and on a combination of leaves) may also contribute to hide much of the intra- or between-leaf variability that is inherent to CCM-200 or conventional fluorescence equipments.

All indicators tested revealed a clear and consistent evolution in time at the 13mM concentration, suggesting the value of that concentration as a reference condition. Among the concentrations below 6.5 mM (0.41, 0.81, 1.63 & 3.25 mM), it is the 0.81 mM treatment which yielded the most stable morphological, biochemical and physiological indicators. At 0.81 and 13 mM, plants showed rapid differences in the indicators tested after the onset of the treatments. These two concentrations have also been chosen by other laboratories of the EU-FP7 project RootOPower

(2012-2015). Therefore, the concentrations for the large scale phenotyping experiments were chosen as 13mM for the control treatment and 0.81mM concentration for the nitrogen deficiency treatment.

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Authors' contributions

G. Lequeue performed experiments, analyzed data and drafted the manuscript. X. Draye and J.-P. Goffart helped to design the study and drafted the manuscript. All authors read and approved the final manuscript.
Conflict of interests

The authors declare no competing financial interests. The views and opinions expressed in this article are purely those of the writers and may not in any circumstances be regarded as stating an official position of the European Commission.

Chapter 5

A genetic analysis of rootstock contribution to biomass production of tomato (*Solanum lycopersicum* L.) under nitrogen deficiency.

In this chapter, high throughput phenotyping was carried out to characterize a rootstock segregating population under deficient and high nitrogen supplies. The phenotyping data included root morphology, plant biomass and nitrogen content evolution. Morphological variables were used to estimate the parameters of a root architecture model and simulate virtual root systems that are realistic representations of experimental root systems. Afterwards, the genetic variability of the different traits considered during the experiment and the characteristics that influence the performance of the rootstock was conducted, in search for QTLs. This approach was used to identify the main strategies that confer resistance under nitrogen deficiency in this rootstock segregating population.

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Article project.

91

Abstract

Nitrogen (N) is the most abundant mineral element of plants and its availability can be a major factor limiting biomass production. The modulation of root system architecture as a function of nitrogen supply and demand is one of the most substantial phenomenon by which plants maximize the chances of reaching maturity (Kraiser et al, 2011). Due to the increasing awareness of their contribution to plant performance in suboptimal conditions, root systems have become new target for several research program upstream of breeding (e.g. DROPs, RootOPower and EuRoot). However, root systems remain difficult to observe in situ. The aim of our study was to dissect the genetic variability of root system architecture of tomato rootstocks under low and high nitrogen supply. A segregating population of 144 recombinant inbred lines (RIL) generated from Solanum lycopersicum var. cerasiforme and S. pimpinellifolium has been used for the QTL analysis of root system architecture and biomass production in a novel aeroponic root phenotyping platform. This material revealed interesting shoot responses to the root genotype, suggesting the validity of the approach. QTLs were detected for three major processes which shape root system architecture, viz. root growth rate, root branching and root tropism. In conclusion, tomato breeding may consider to modify root system architecture towards ideotypes that would be suitable to specific environments or crop management systems.

5.1. Introduction

Ensuring an adequate supply of nutrients under fluctuating and sometimes stressful environmental conditions is one of the most significant challenges for plants. As nitrogen (N) is the most abundant mineral element in plant tissues, low nitrogen availability is often a limiting factor of biomass production (Tremblay *et al*, 2012; Goffart *et al.*, 2013; Ben Abdallah *et al*, 2016). Soil nitrogen is part of a large biogeochemical cycle, and comprises different chemical forms which are not evenly available for the plant. In addition, one of those forms, nitrate, has a high mobility in the soil and is susceptible to escape the root zone, leach to deep soil layers and accumulate in the ground water (Kraiser *et al.*, 2011). Therefore, although it difficult to predict the amount of nitrogen available and accessible to plants in natural environments and in field conditions, it essential for the plant, the farmer and the environment to ensure efficient nitrogen capture. The adjustment of physiology, growth and development of plant root systems as a function of nitrogen supply comprise morphological responses to match root placement with nitrogen distribution as well as modifications of the nitrogen transport systems.

The modulation of root system architecture is one of the most substantial adaptations used by plants to ensure adequate nitrogen acquisition (Kraiser *et al.*, 2011). The mechanisms involved are not yet fully understood, but important elements have been identified, which refer to the membrane transport of auxin and abscisic acid (ABA), both involved in the growth and branching of root (Signora *et al.*, 2001; Forde, 2002; Walch-Lui *et al.*, 2006b; Tian *et al.*, 2008; Bellini *et al.*, 2014). In barley (*Hordeum sativum* L.), the early studies of Drew *et al.* (1973) and Drew (1975) have shown that a localized supply of nitrate or ammonium leads to an increase proliferation of lateral roots in the nutrient rich zone. This response occurs in many

plant species and represents a common adaptation mechanism (Robinson, 1994; Hodge, 2004). Responses to nitrogen also includes changes in primary root growth (Walch-Liu *et al.*, 2006b; Walch-Liu and Forde, 2008; Vidal *et al.*, 2010a), lateral root initiation (Little *et al.*, 2005; Remans *et al.*, 2006b; Gifford *et al.*, 2008), and lateral root elongation (Zhang and Forde, 1998; Zhang *et al.*, 1999; Vidal *et al.*, 2010a). The general view is that primary root and lateral root elongation are inhibited in uniform and high nitrogen supply, while lateral root elongation and initiation are stimulated in nitrogen rich areas of patchy environments.

The yield and quality of grafted tomato depend on the characteristics of rootstocks (Lee and Oda, 2003; Davis *et al.*, 2008a; Salehi-Mohammadi *et al.*, 2009; Flores *et al.*, 2010; Rouphael *et al.*, 2010; Schwarz *et al.*, 2010; Savvas *et al.*, 2011; Geboloğlu *et al.*, 2011; Goto *et al.*, 2013; Krumbein and Schwarz, 2013; Schwarz *et al.*, 2013; Al-Harbi *et al.*, 2016). Rootstocks can provide stability and tolerance against to chilling, suboptimal and supraoptimal temperature (Rivero *et al.*, 2003a; Rivero *et al.*, 2003b; Schwarz *et al.*, 2010; Ntatsi *et al.*, 2013; Ntatsi and Savvas, 2014), salt stress (Rivero *et al.*, 2003b; Estan *et al.*, 2004; Martinez-Rodriguez *et al.* 2008; Albacete *et al.*, 2015a), nutrient stress (Rivero *et al.*, 2003b; Davis *et al.*, 2008; Savvas *et al.*, 2011; Schwarz *et al.*, 2013; Albacete *et al.*, 2015b; Al-Harbi *et al.*, 2016), drought (Keatinge *et al.*, 2014; Al-Harbi *et al.*, 2016; Yao *et al.*, 2016) and soil borne diseases (Rivero *et al.*, 2003b; Louws *et al.*, 2010; Foolad and Panthee, 2012; Borgognone *et al.*, 2013; Keatinge *et al.*, 2014).

As a result, tomato breeding has focused on the production of new rootstocks which achieve better tolerance to diseases and abiotic stresses as well as efficiency of water and nutrient uptake. Conventional breeding is based on the empirical evaluation of new genotypes in environments which the target performance is best expressed. Recently, novel strategies combining modelling, phenotyping and quantitative genetics have proven useful to improve water, phosphorus and nitrogen capture (Lynch, 2011). These strategies, which integrate knowledge of the ecophysiological mechanisms (phenes) underlying the target performance (phenotype) and of their genetic bases, lead to the prediction of performance of novel genotypes and to the design new ideotypes (Lynch, 2013). They have been particularly well developed for root traits which are difficult to handle in conventional breeding (Rich and Watt, 2013).

In this context, root system architecture has received increasing attention from the scientific community (*e.g.* the DROPS, EURoot and RootOPower projects). The concept of architectural analysis is based on the description of root dynamics (*e.g.* growth, branching tropisms and senescence) and their hierarchical relationships over time (Hallé and Oldeman, 1970). This analysis leads to define a limited number of root types based on structure and behavior. By quantifying dynamics for each root types, one is able to simulate root system architecture over time and to evaluate in silico the ability of virtual genotypes to capture resources in their environment (Jourdan and Rey, 1997; Smit *et al.*, 2000; Dunbabin *et al.*, 2003; Vercambre *et al.*, 2003; Pagès *et al.*, 2004; Draye *et al.*, 2010; Dunbabin *et al.*, 2013).

The present study aims at better considering the interactions between shoot and roots in strategies for improving nitrogen capture. For this purpose, root system dynamics and nitrogen content have been evaluated in a segregating population of rootstocks contrasting in low nitrogen tolerance, which were grafted to the same scion and grown under low and high nitrogen in an aeroponics platform.

5.2. Materials and methods

5.2.1. Plant material and grafting

A commercial tomato cultivar (*Solanum lycopersicum* L. 'Boludo F1', 'Monsanto') was grafted onto 145 different rootstocks: six accessions from *S. lycopersicum* ('*Cerasiforme*') and *S. pimpinellifolium*, selected for drought tolerance (sourced from The World Vegetable Center, AVRDC); nine introgression lines from *S. lycopersicum* × *S. pennellii* and × *S. habrochaites*, selected for high root/shoot ratio, salinity and drought tolerances (sourced from The Tomato Genetics Resource Center, TGRC); a population of 129 recombinant inbred lines (RILs) derived from a salt sensitive genotype of *S. lycopersicum* var. *cerasiforme* and a salt tolerant line from *S. pimpinellifolium* L. (Monforte *et al.*, 1997) sourced from Instituto Valenciano de Investigaciones Agrarias (IVIA) and Boludo F1 (self-graft). The RIL population had been developed in previous studies to analyze the rootstock-mediated variation in tomato vegetative growth under low potassium, low phosphorous, drought, salinity and soil impedance stresses (Albacete *et al.*, 2015a; Albacete *et al.*, 2015b) and to perform other genetic studies (Asins and Carbonell, 2014; Asins *et al.*, 2015; Kevei *et al.*, 2015).

Grafting was performed using the splicing method at the 2-3 true leaf stage (3-4 weeks after sowing), and the scion was attached at the first node of the rootstock. One month after grafting, the primary and adventitious roots were excised. Plants were stored (for maximum one week) in individually sealed plastic bags containing a piece of wet blotting paper and kept in the dark in an air-conditioned room at 12°C before the start of the phenotyping experiment (Kubota and Kroggel, 2006).

5.2.2. Plant growth conditions in greenhouse

Three plants per genotype were transferred in each of two aeroponic systems for a one-week long acclimation period under low light and high humidity. Each system was supplied with 75 L of a modified Hoagland Solution (Hoagland and Arnon, 1950) with a nitrogen concentration of 13 mmol.l⁻¹ (in mmol.L⁻¹: NO3⁻ 12.00; NH4⁺ 1.00; H2PO4⁻ 2.01; SO4²⁻ 1.00; K⁺ 6.01; Ca²⁺ 3.50; Mg²⁺ 1.00; plus 0,238 g.L⁻¹ of an EDTA-microelements complex, pH = 5.6, EC = 1.6 dS/m). After acclimation, one of the systems was supplied with a low nitrogen concentration of 0.81 mmol.l⁻¹ (in mmol.L⁻¹: NO3⁻ 0.75; NH4⁺ 0.06; H2PO4⁻ 2.01; SO4⁻ 6.16; K⁺ 6.01; Ca²⁺ 3.50; Mg²⁺ 1.00; plus 0,238 g.L⁻¹ of an EDTA-microelements complex, pH = 5.6, EC = 1.5 dS/m) and the other one was maintained with the initial nitrogen concentration of 13 mmol.l⁻¹. The control and low nitrogen solutions were renewed three times per week and the experiment lasted 17 days. The full experiment was performed three times. The first assay started on 31 January 2014, the second on 7 March 2014 and the third on 4 April 2014.

A data-logger (Aria Supervisor, A.R.I.A., Neung sur Beuvron, France) recorded greenhouse climate data from the beginning of the application of treatments (control and low nitrogen): mean daily air temperature for each experiments was, respectively, 20.6 °C (max. 25.8/22.5 °C day/night, min. 15.0/14.0 °C day/night), 21.7 °C (max. 29.1/25.5 °C day/night, min. 16.5/14.9 °C day/night), 21.1 °C (max. 29.0/27.0 °C day/night, min. 17.9/18.9 °C day/night) and mean daily relative humidity for each experiments was, respectively, 74.0 % (max. 85.0/86.0 % day/night, min. 35.0/39.0 % day/night), 75.1 % (max. 94.0/91.0 % day/night, min. 39.0/51.0 % day/night), 70.0 % (max. 88.0/90.0 % day/night, min. 34.0/52.0 % day/night). The photoperiod was set at 16 h during the entire assay period. Solar

radiation was complemented with 9 blue-white-red LED fixtures (Lumigrow Pro 650) located 1.5 m above the plants and providing 150 μ mol m⁻² s⁻¹, measured with a photosynthetically active radiation sensor (PAR Lite, Kipp and Zonen, Delft, The Netherlands) at the top of the plants.

5.2.3. Plant biomass measurements

Shoot and roots, fresh and dry biomass (after oven-drying at 65°C until constant weight) were also determined.

5.2.4. Optical measurements

Fluorescence emitted by plant pigments after excitation has been recorded *in situ* using the Multiplex ResearchTM hand-held multi-parameter fluorescence sensor (FORCE-A, Orsay, France). The instrument generates excitation fluorescence in four channels (UV 375 nm, blue 470 nm, green 515 nm, red 625 nm) in a three-second long sequence of 250 flashes and records the emitted fluorescence of the exposed leaves (5000 mm²). The 20 parameters (*e.g.* Fluorescence-Excitation Ratios,...) derived from this acquisition are used to estimate the concentration of anthocyanin content, flavonol content, chlorophyll content, or as indicators of the plant physiological status (such as ANTH-RG, FER_RG, FLAV, SFR-G, NBI-R, FERARI). More details about the sensor can be found in Ben Ghozlen *et al.* (2010), Tremblay *et al.* (2012), the user guide (Force-A, 2017) and chapter 4. Multiplex measurements started at the onset of the nitrogen treatments and were conducted between 9:00 and 12:00 on each plant every three days. For each plant, the evolution of the NBI-R indicator over time was computed as the slope of a linear regression of NBI-R on time.

5.2.5. Root structure architecture parameters evaluation

After excision of the primary and adventitious roots of the grafted plants, the plants started to emit a variable number of adventitious roots from the base (rootstock section) of the shoot. The architectural analysis was made on individual root system images acquired at the end of the experiment and evaluated the number and tropism of adventitious and lateral roots. Due to the large density of roots on individual images and to the large number of images (ca. 2700) to process, empirical proxys of architectural parameters were defined and estimated either manually or using custom-made macros in the ImageJ (2017) software. Images were first registered using the TurboReg plugin (Thévenaz *et al.*, 1998) and thresholded to generate binary images where foreground pixels (white) correspond to root objects. After registration, the pixel size was 35.7 microns, corresponding to a scanning resolution of ca. 700 DPI.

The *Root density* was determined as the proportion of foreground pixels in a rectangular region (5 cm x 3.5 cm) of the root system located between 5 and 10 cm below the stem base.

The insertion angle of adventitious roots and lateral roots was estimated manually along a scale of five angle categories (from 0...18° to 72...90°) coded 1 (the root follows the longitudinal axis of the parent organ) to 5 (the root emerges perpendicular to the longitudinal axis of the parent organ). Only roots growing parallel to the plane of the image were considered for this evaluation, to avoid the biais due to the 2D projection. *The average spacing between lateral roots* was estimated visually along a categorical scale with a 5 mm resolution.

The number of adventitious roots was determined in two regions located left and right to the stem, considering only the portion of the stem from which adventitious roots were growing. The regions were defined as 500-pixel wide parallelograms, with a near-to-vertical side following the edge of the stem and an oblique side parallel to the growth direction of adventitious roots. The number of foreground pixels in these regions was divided by the adventitious root thickness (in pixels) and by the width of the region. The resulting value, rounded to the closest integer, was considered as a proxy of the number of adventitious roots.

The *horizontal width of the root system* was estimated as the distance between the edges of the root system 4 cm below the top of the root system. The edge foreground pixels were detected on eleven horizontal lines (between 3.9 and 4.1 cm below the top of the root system). The horizontal width was computed from the difference between the median horizontal position of the left and right edge pixels.

Additional parameters were obtained using the RootTyp simulation model (Pagès *et al.*, 2004) and an inverse modelling strategy described in Chapter 6 of the thesis. This strategy is based on finding RootTyp parameter values which maximize the match between simulated and observed root system images.

5.2.6. Statistical analysis

Statistical analyzes was performed using the Statistical Analysis System (SAS) software version 9 (SAS Institute Inc, Raleigh, North Carolina, United States). Twoway variance analyses were performed, with experiment and nitrogen treatment as fixed effects, in order to detect treatment x experiment interactions. From the results of these analyses, it was decided to adjust individual values for spatial (within the aeroponics system) and temporal (across experiments) variability. Adjusted values were used in two-way mixed model variance analyses with line as random effect and treatment as fixed effect, to estimate the genotypic (Vg) and error variances (Vr). Broad-sense heritabilities were then obtained at the global level as $H^2 = Vg/(Vg + Vr/n)$, where n is the number of measurements per line. Heritabilities were also estimated separately for each nitrogen treatment, using variance components of the mixed model analysis performed separately for each treatment.

5.2.7. QTL analysis

Adjusted phenotypic line means were used in combination with 7720 SNPs genotypic data (Asins *et al.*, 2015) to carry a QTL analysis using Interval Mapping (IM) and Multiple QTL Mapping (MQM) procedures in MapQTL® 6 (Van Ooijen, 2009). A 5 % experiment-wise significance level was assessed by permutation tests. These LOD critical values ranged from 1.70 to 2.47 depending on the trait and chromosome.

5.3. Results and discussion

5.3.1. Phenotyping data

The morphological diversity observed in this large scale phenotyping experiment (ca. 2700 plants) is illustrated on **Figure 5-1**. This diversity concerned root length, numbers and angles. Due to the fact that all lines had been grafted on a common scion, we can be confident that the observed differences arise from regulatory

processes that are initiated in the root system. The raw datasets included (i) a number of morphological descriptors obtained from direct or image-based measurements on individual plants as well as (ii) parameters derived from the inverse modelling strategy (**Table 5-1**).



Figure 5-1. The morphological diversity of root systems in the aeroponic platform (top left: narrow branching angle of adventitious roots; top center: large branching angle of adventitious roots; top right: plagiotropic lateral roots; bottom left: lack of tropism; bottom center: long lateral roots; bottom right: large number of adventitious roots).

As differences between experiments were noticeable, we first tested for experiment x treatment interactions in a two-way variance analysis (**Table 5-2**). The interaction was significant for biomass production (root and shoot dry weight), and for geometrical aspects of root architecture (angle, width and density), but was not

Chapter 5

significant for adventitious root growth and development (length and number). Presumably, the growth of lateral roots, which was not recorded here, and the distance between laterals, that are highly responsive to the root environment and plant status, contributed to the significant interaction for root dry mass.

Table 5-1. List of traits evaluated under optimal (C) and low N (N) and trait code for QTL analysis in the experiment by UCL.

Trait code	ode Trait					
Sfw	Shoot Fresh Weight					
Sdw	Shoot Dry Weight					
Rfw	Root Fresh Weight					
Rdw	Root Dry Weight					
Exprdw	exp(rdw)					
Mrl	Maximum Root Length					
RootDens	Root Density (based on image pixel frequency)					
RootNb	Adventitious Root Number (based on image pixel frequency)					
InterLR	Distance between successive Lateral Roots (manual score)					
RootAngle	Adventitious Root Angle (manual score)					
LRAngle	Lateral Root Angle (manual score)					
HWidth	Horizontal width (manual measurement)					
RootDens2	Model-based estimation of Root Density					
RootNb2	Model-based estimation of Adventitious Root Number					
RootAngle2	Model-based estimation of Adventitious Root Angle					
Angle2	Model-based estimation of Adventitious Root Angle (variant method)					
InterLR2	Model-based estimation of the Inter Lateral Distance					
Hwidth2	Model-based estimation of Horizontal width					

 Table 5-2: Evaluation of interaction between the repetition of the experiment and treatment.

Troit	P-value of the interaction test			
Shoot Fresh Weight	<0.001			
Shoot Dry Weight	<0.001			
Root Fresh Weight	0.1168			
Root Dry Weight	<0.001			
Maximum Root Length	0.0788			
Root Density	<0.001			
Adventitious Root Number	0.8932			
Distance between Lateral Roots	0.015			
Adventitious Root Angle	<0.001			
Lateral Root Angle	<0.001			
Horizontal width	<0.001			

The distributions of genotypic means for the adjusted variables, under control *vs* low N conditions, are illustrated on **Figures 5-2** and **5-3**. They reveal a large genotypic variation for all variables, except for the shoot fresh weight whose genotypic differences vanished under low N. Figures 5-2 and 5-3 allow to disentangle the different responses of the RIL population to low N.

Shoot wet and dry weights were reduced by low N, yet only for the lines that had a large shoot biomass under high N. Under low N, all these lines reduced their shoot biomass to the level of the lines with low shoot biomass. For these low production lines, shoot biomass was not affected by low N. Therefore, it might seem more appropriate to say that the lines differed in their ability to take benefit of high nitrogen supply, than to express these results in terms of tolerance to low N. This interpretation is line with the evolution of N content that was enhanced under high N in a large number of lines, but that was not affected for the remaining lines. Interestingly, there was no correlation between N evolution in low N *vs* high N conditions, and a small number of lines had an improved N evolution in low N than in high N.

The length of the longest root and the root dry weight were increased under low N, but only in the lines that had already a superior root length or dry weight in high N. It is possible that lines with a superior root length had larger N requirements or were more dependent on adventitious root length to acquire their nitrogen and have an advantage to increase root length under low N. An interesting result is the difference of correlation between maximum root length and root fresh weight (taken as a proxy of root volume) under low and high N conditions. Under high N, root fresh weight ranged from ~2 to ~8 gr, maximum root length ranged only from ~60 to ~90 cm and there was no obvious correlation between the two variables, suggesting that adventitious root number or lateral root traits contributed largely to the variation of root volume.





Figure 5-2. Distribution of genotypic means in low and high N conditions (I).

Under low N, however, the range of maximum root length was extended (~60 to ~130 cm) and the correlation with fresh weight was increased, indicating that the variation of root volume under low N was largely determined by variations of root volume. This suggests that low N may cause a modification of N capture strategy.



Figure 5-3. Distribution of genotypic means in low and high N conditions (II).

Finally, the number of adventitious roots tended to decrease under low N for many lines, and the horizontal width of a few lines were increased under low N (Figure 5.3).

Taken together, these distributions indicate that most aspects of root system growth and development are somehow involved in the response to low N. A systemic analysis of this dataset should be performed in order to relate N uptake performance under low N with the variation of root architecture. However, given the low accuracy of the root architecture proxies that were generated here, we did not embark in this type of complex analysis.

5.3.2. Heritability

Heritabilities estimated from adjusted line means are listed in **Table 5-3**. With few exceptions, the broad-sense heritabilities (H2) values for the variables were reasonably high and similar when estimated for each treatment or globally. The highest heritabilities were obtained for variables that are indicative of shoot or root system size (length and weight).

Low N High N Global Response **Shoot Fresh Weight** 0.44 0.57 0.45 0.10 Shoot Dry Weight 0.37 0.53 0.47 0.00 0.00 **Root Fresh Weight** 0.60 0.61 0.61 0.41 **Root Dry Weight** 0.37 0.38 0.00 0.10 0.61 0.56 Maximum Root Length 0.63 0.32 0.13 0.28 0.00 **Top Root Density** Adventitious Root Number (model) 0.10 0.29 0.25 0.00 **Inter Lateral Root Distance** 0.37 0.38 0.00 0.00 0.06 0.31 0.21 0.09 Adventitious Root Angle (model) Adventitious Root Angle (model - variant) 0.10 0.23 0.15 0.00 Horizontal Width (model) 0.47 0.42 0.32 0.18

Table 5-3. Broad sense heritabilities, computed separately for each N supply (low and high N), at the experiment-wise level (Global) and for the response to N supply (Response).

Moderate to low heritabilities were obtained for the adventitious root number and angle. These low values are rather surprising given the large heritability of root fresh

weight and horizontal width, to which root number and angle are expected to contribute. Finally, the complete loss of heritability for inter-lateral root distance when one compares the low or high N heritabilities with the global heritability suggests that the differences of distance among lines in low N have different genetic causes than those that are seen in high N.

5.3.3. QTL analysis

In contrast with the heritability values, a rather small number of QTL were detected, all of which having small LOD score values, between 1.75 and 2.67 (**Table 5-4**). By comparison, the LOD scores of root weight QTLs obtained in other experiments using this plant population ranged between 2.03 and 3.99 (MJ Asins, pers. com.). As expected, no QTLs were detected for variables with small broad sense heritabilities.

QTLs were detected for maximum root length (mrl), root branching (InterLR) and root tropism (HWidth). These relate to three major processes which shape root system architecture. Interestingly, four additional QTLs (not co-locating with the previous ones) were observed for root biomass and root density. These are likely to represent root number or root diameter effects that were not (or not accurately) captured by the phenotyping pipeline.

Under low N, a reasonably good fit regarding the position and direction of allelic effects was found between manual and model-based estimates of root branching (InterLR and InterLR2, two QTLs on Chr 6 and 9) and of root system width (HWidth and HWidth2, two QTLs on Chr 2 and 9). An additional QTL was detected for the model-based estimate of root branching (InterLR2_N on Chr6).

Chapter 5

Table 5-4. List of QTLs detected by interval mapping (5% overall significance level). The map position (Position) of QTL peaks in the *Solanum pimpinellifolium* chromosome (Group) is indicated in cM. The genotypic means for *escullentum* (mu_A) and *pimpinellifolium* (mu_B) homozygotes, the estimated additive value (Additive) and the percentage of explained variance (% Expl.) are also shown.

Trait	Group	Position	LOD	mu_A	mu_B	% Expl.	Additive
sdw_C	P11	16.131	2.46	4.14	4.00	8.3	0.07
rfw_C	P2	32.414	1.91	4.56	3.97	6.5	0.29
rdw_N	P2	42.120	1.91	2.87	2.84	6.6	0.02
rdw_N	P9	3.000	1.82	2.87	2.84	6.3	0.01
exprdw_N	P2	41.120	2.02	17.81	17.22	7.0	0.29
exprdw_N	P9	2.000	1.70	17.75	17.26	5.9	0.24
mrl_N	P6	29.723	1.83	93.28	82.26	6.4	5.51
RootDens_ C	P1b	60.655	2.08	0.57	0.53	7.1	0.02
interLR_C	P2	16.773	1.85	2.07	1.82	6.4	0.12
interLR_N	P9	56.809	1.99	2.11	1.90	6.9	0.10
HWidth_N	P6	47.186	1.81	2109.15	1954.54	6.3	77.30
HWidth_N	P9	53.809	1.91	1963.45	2116.38	6.6	-76.46
InterLR2_C	P2	16.773	2.37	0.79	0.69	8.1	0.05
InterLR2_N	P6	0.000	1.97	0.68	0.76	6.8	-0.04
InterLR2_N	P9	57.237	2.27	0.77	0.68	7.9	0.05
HWidth2_N	P6	40.792	2.67	7.30	6.70	9.2	0.30
HWidth2 N	Р9	51.809	1.75	6.77	7.31	6.1	-0.27

5.4. Conclusion

In this phenotyping study, an important morphological diversity was observed within the grafted populations. This diversity appears to involve all developmental and growth processes underlying root system architecture. Interestingly, the heritabilities for the inter-lateral distance indicates that the genetic determinism of lateral root initiation is largely different at low and high nitrogen conditions. This is an important result as lateral roots are known to respond to N supply and plant N status and contribute to much of the absorptive surface of the root system. Surprisingly, the heritability of the response of inter-lateral distance to N supply was negligible, indicating that the genetic determinism of N response is complex.

A classical observation in low N conditions is an increase in root length and a decrease of fresh weight which leads to an increase of the specific length of roots (Miller and Cramer, 2005). This implies a redistribution of the root volume from the top profile to enable faster root growth at depth. When extended to a whole segregating population, the responses to low N appear to be much more diverse and complex that what has been reported in the literature. This result calls for systematic analysis of detailed responses in large panels of genotypes, in order to identify promising root foraging strategies.

QTLs were observed for three major processes which shape root system architecture, root growth rate, root branching and root tropism. In addition, low LOD scores in aeroponics have been commonly observed in maize, barley, wheat and rice (in our laboratory). This suggests that, in the absence of constraints, large collections of genotypes within a species tend to achieve a narrow range of phenotypes. In view of very large QTL effects reported in the literature in soil or other conditions, this would indicate that the scope for root architecture improvement lays more in the root responses to environmental constraints than on constitutive traits. If this was to be the case, the scientific community should probably reconsider much of the actual root architecture phenotyping practices.

At this stage, the whole experimental and analysis pipeline allows to reproduce a gross trend across the population and N levels, however, the limited value of the validation suggests that this information should not be trusted to predict details of the root architecture of individual genotypes. We anticipate that the quality of the

phenotyping would be dramatically enhanced with an improved chain of image capture and analysis, which has been recently implemented in the aeroponics platform.

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Authors' contributions

G. Lequeue performed experiments, analyzed data and drafted the manuscript. B. De Leener helped to analyzed data. X. Draye and M.-J. Asins helped to design the study and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflicts of interest. The views and opinions expressed in this article are purely those of the writers and may not in any circumstances be regarded as stating an official position of the European Commission.

112

Chapter 6

Model-based conception of root traits of tomato (*Solanum lycopersicum* L.) rootstocks.

In this chapter, a model-assisted image analysis pipeline was developed to determine root traits which respond to nitrogen supply. The morphogenetic characteristics obtained in the previous chapter were used to evaluate the pipeline.

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Article project.

113

Abstract

The modulation of root system architecture is the most substantial adaptation of plants to ensure an adequate nitrogen supply. The analysis of architectural responses is however fraught with practical difficulties related to root observation and architecture quantification. The development of dynamic models of root system architecture has led to the identification of few critical parameters which can be used to capture changes of root growth and development. The aim of this study was to develop a model-assisted image analysis pipeline to determine root traits which respond to nitrogen supply. A rootstock segregating population of 144 recombinant inbred lines (RIL) generated after a cross between Solanum lycopersicum var. cerasiforme and S. pimpinellifolium has been used in a large scale root phenotyping experiment under control (13 mmol.l⁻¹) and nitrogen deficient (0.81 mmol.l⁻¹) conditions in a novel aeroponic platform. The phenotyping data were used to estimate model parameters and simulate virtual root systems that are realistic representations of experimental root systems for many of the 144 lines in both nitrogen conditions. The correlation between simulated and measured root mass has been estimated to assess the accuracy of the pipeline.

6.1. Introduction

Nitrogen is the first of three essential plant macronutrients, followed by potassium and phosphorus (Jia *et al.*, 2008, Bellini *et al.*, 2014). It is also the element that has the most impact on growth, due to its being a major constituent of amino acids and proteins that are essential for metabolism, growth and development. Nitrogen represents 2.7 to 5% of the dry weight of tomato (Taiz and Zieger, 2010). It is found in higher concentrations during the early stages of development (Taiz and Zieger, 2010). Under nitrogen deficiency, tomato growth is reduced and its life cycle is shortened, probably as a consequence of a decrease in the synthesis and turnover of proteins involved in energy metabolism such as respiration and photosynthesis (Shin *et al.*, 2005; Taiz and Zieger, 2010).

In tomato plants sown in the field, the primary root normally develops into a thick central taproot, which develops lateral roots which reiterate the branching process by forming laterals roots of higher orders (Bellini *et al.*, 2014). This root system architecture is defined as allorhizic (Devaiah *et al.*, 2007). In such plants, adventitious roots are rare, but can emerge from the stem, especially in case of damage (Osmond *et al.*, 2007). However, after transplanting, which is a common operation in tomato production, the taproot is generally highly disturbed (Picken *et al.*, 1986) and the plants develop a new root system based on new adventitious roots. The mechanisms leading to the formation of adventitious roots are not fully understood, but there is increasing evidence that these mechanisms share common pathways with those leading to the formation of lateral roots (Orman-Ligeza *et al.*, 2013).

Plants have evolved several strategies to manage the scarcity of the nitrogen resource, many of which aim at promoting the absorption of the element. The modulation of root system architecture is one of the most substantial plant adaptations to ensure an adequate nitrogen acquisition (Kraiser *et al.*, 2011). An increase in root-shoot ratio is usually achieved in situations of nitrogen deficiency (Chapin III *et al.*, 1993; Miller and Cramer, 2005). This increase is due to a reduction of shoot growth more than an increase in root growth (Jackson and Bloom, 1990). This relative promotion of root growth likely reflects a higher priority in the allocation of nitrogen to roots (Miller and Cramer, 2005). To increase the absorption of nitrogen, plants may also promote root growth and formation within nitrogenrich zones (Hetrick, 1991; Miller and Cramer, 2005). The mechanisms involved are not yet fully understood, but the plant hormones auxin and abscisic acid appear to participate in the coordination of root growth according to the status of nitrogen (Miller and Cramer, 2005; Bellini 2014). The number and distribution of root hairs are also decisive factors in the absorption of nitrogen (Miller and Cramer, 2005).

Although roots are considered an essential part of the plant, their features remain very difficult to observe and quantify. The development of new techniques has allowed to acquire dynamic, accurate and non-invasive data on root architecture which pave the way for addressing root growth and development from the organ to the plant scale. Structural models like RootTyp (Pagès *et al.*, 2004) or functional structural plant models like PlaNet (Lobet *et al.*, 2012) are available to evaluate in silico the benefits of different root system behaviors and better understand the coordination of organs growth and metabolism within the plant and as a function of their environment (Fourcaud *et al.*, 2008; Guo *et al.*, 2011). The parameters of these models capture underlying causes of root system architecture and provide alternative descriptors of root systems.

The objectives of the present study was to develop and evaluate a novel data analysis pipeline to estimate the values of several RootTyp parameters in high throughput phenotyping experiments in tomato, as an alternative of classical and timeconsuming tracing of root architecture.

6.2. Materials and methods

6.2.1. Plant material and root structure architecture parameters in situ evaluation

The experimental conditions, techniques and root structure architecture parameters obtained with aeroponics culture system have been previously described in Chapters 5. A commercial tomato cultivar (Solanum lycopersicum L. 'Boludo F1', 'Monsanto') was grafted onto 145 different rootstocks: six accessions from S. lycopersicum ('Cerasiforme') and S. pimpinellifolium, selected for drought tolerance (sourced from The World Vegetable Center, AVRDC); nine introgression lines from S. lycopersicum × S. pennellii and × S. habrochaites, selected for high root/shoot ratio, salinity and drought tolerances (sourced from The Tomato Genetics Resource Center, TGRC); a population of 129 recombinant inbred lines (RILs) derived from a salt sensitive genotype of S. lycopersicum var. cerasiforme and a salt tolerant line from S. pimpinellifolium L. (Monforte et al., 1997) sourced from Instituto Valenciano de Investigaciones Agrarias (IVIA) and Boludo F1 (self-graft). The RIL population had been developed in previous studies to analyze the rootstock-mediated variation in tomato vegetative growth under low potassium, low phosphorous, drought, salinity and soil impedance stresses (Albacete et al., 2015a; Albacete et al., 2015b) and to perform other genetic studies (Asins and Carbonell, 2014; Asins et al., 2015; Kevei et al., 2015). Grafting was performed using the splicing method at the 2-3 true leaf stage (3-4 weeks after sowing), and the scion was attached at the first node of the rootstock. One month after grafting, the primary and adventitious roots were excised. Plants were stored (for maximum one week) in individually sealed plastic bags containing a piece of wet blotting paper and kept in the dark in an airconditioned room at 12°C before the start of the phenotyping experiment (Kubota and Kroggel, 2006).

Three plants per genotype were transferred in each of two aeroponic systems for a one-week long acclimation period under low light and high humidity. Each system was supplied with 75 L of a modified Hoagland Solution (Hoagland and Arnon, 1950) with a nitrogen concentration of 13 mmol.l⁻¹ (in mmol.L⁻¹: NO3⁻ 12.00; NH4⁺ 1.00; H2PO4⁻ 2.01; SO4²⁻ 1.00; K⁺ 6.01; Ca²⁺ 3.50; Mg²⁺ 1.00; plus 0,238 g.L⁻¹ of an EDTA-microelements complex, pH = 5.6, EC = 1.6 dS/m). After acclimation, one of the systems was supplied with a low nitrogen concentration of 0.81 mmol.l⁻¹ (in mmol.L⁻¹: NO3⁻ 0.75; NH4⁺ 0.06; H2PO4⁻ 2.01; SO4⁻ 6.16; K⁺ 6.01; Ca²⁺ 3.50; Mg²⁺ 1.00; plus 0,238 g.L⁻¹ of an EDTA-microelements complex, pH = 5.6, EC = 1.5 dS/m) and the other one was maintained with the initial nitrogen concentration of 13 mmol.l⁻¹. The control and low nitrogen solutions were renewed three times per week and the experiment lasted 17 days. The full experiment was performed three times. The first assay started on 31 January 2014, the second on 7 March 2014 and the third on 4 April 2014.

A data-logger (Aria Supervisor, A.R.I.A., Neung sur Beuvron, France) recorded greenhouse climate data from the beginning of the application of treatments (control and low nitrogen): mean daily air temperature for each experiments was, respectively, 20.6 °C (max. 25.8/22.5 °C day/night, min. 15.0/14.0 °C day/night), 21.7 °C (max. 29.1/25.5 °C day/night, min. 16.5/14.9 °C day/night), 21.1 °C (max. 29.0/27.0 °C day/night, min. 17.9/18.9 °C day/night) and mean daily relative humidity for each experiments was, respectively, 74.0 % (max. 85.0/86.0 % day/night, min. 35.0/39.0 % day/night), 75.1 % (max. 94.0/91.0 % day/night, min. 39.0/51.0 % day/night), 70.0 % (max. 88.0/90.0 % day/night, min. 34.0/52.0 % day/night. The photoperiod was set at 16 h during the entire assay period. Solar radiation was complemented with 9 blue-white-red LED fixtures (Lumigrow Pro 650)

located 1.5 m above the plants and providing 150 μ mol m⁻² s⁻¹, measured with a photosynthetically active radiation sensor (PAR Lite, Kipp and Zonen, Delft, The Netherlands) at the top of the plants.

After excision of the primary and adventitious roots of the grafted plants, the plants started to emit a variable number of adventitious roots from the base (rootstock section) of the shoot. The architectural analysis was made on individual root system images acquired at the end of the experiment and evaluated the number and tropism of adventitious and lateral roots. Due to the large density of roots on individual images and to the large number of images (ca. 2700) to process, empirical proxys of architectural parameters were defined and estimated either manually or using custom-made macros in the ImageJ (2017) software. Images were first registered using the TurboReg plugin (Thévenaz *et al.*, 1998) and thresholded to generate binary images where foreground pixels (white) correspond to root objects. After registration, the pixel size was 35.7 microns, corresponding to a scanning resolution of ca. 700 DPI.

The *Root density* was determined as the proportion of foreground pixels in a rectangular region (5 cm x 3.5 cm) of the root system located between 5 and 10 cm below the stem base.

The insertion angle of adventitious roots and lateral roots was estimated manually along a scale of five angle categories (from 0...18° to 72...90°) coded 1 (the root follows the longitudinal axis of the parent organ) to 5 (the root emerges perpendicular to the longitudinal axis of the parent organ). Only roots growing parallel to the plane of the image were considered for this evaluation, to avoid the biais due to the 2D projection. *The average spacing between lateral roots* was estimated visually along a categorical scale with a 5 mm resolution.

The number of adventitious roots was determined in two regions located left and right to the stem, considering only the portion of the stem from which adventitious roots were growing. The regions were defined as 500-pixel wide parallelograms, with a near-to-vertical side following the edge of the stem and an oblique side parallel to the growth direction of adventitious roots. The number of foreground pixels in these regions was divided by the adventitious root thickness (in pixels) and by the width of the region. The resulting value, rounded to the closest integer, was considered as a proxy of the number of adventitious roots.

The *horizontal width of the root system* was estimated as the distance between the edges of the root system 4 cm below the top of the root system. The edge foreground pixels were detected on eleven horizontal lines (between 3.9 and 4.1 cm below the top of the root system). The horizontal width was computed from the difference between the median horizontal position of the left and right edge pixels.

Shoot and roots, fresh and dry biomass (after oven-drying at 65°C until constant weight) were also determined.

6.2.2. Root growth modelling

We used the simulation model RootTyp developed by Pagès *et al.* (2004) and running in the environment CrossTalk (Draye and Pagès, 2008). This program simulates in 4D (3D + time) the growth of plant root systems made of different root types and allows local soil effects on root growth, branching and growth direction. RootTyp also allows to simulate a stem segment that forms adventitious roots. The parameters of the RootTyp model relate to eight biological processes: root formation and acropetal branching, axial and radial growth, tropisms, reiteration, transition and senescence.

For any given root, axial growth is assumed to decrease over time as the root length approaches a set maximum length. It is multiplied by a soil parameter (for the soil layer where the root tip is located) which can enhance or reduce root growth. The initial growth rate and maximum root length are set for each root type.

The direction of growth is derived from the growth direction at the preceding time step, after addition of deviations due to (i) root-specific tropism type and strength, (ii) random mechanical perturbation by the soil and (iii) soil anysotropy (*e.g.* presence of vertical earthworm galleries). The tropism type and strength and the sensitivity to soil mechanics are set for each root type.

The initial direction of growth of a root is expressed as (i) an insertion angle between the axes of the new root and its parent organ and (ii) an azimuth angle which is always taken from a random uniform distribution. The insertion angle is set for each root type.

Branching in RootTyp is always acropetal. Successive roots are formed with a preset inter-branch distance. The settings of each root type specifies the inter-branch distance and the root type of the new branches.

In our simulations of root-excised tomato rootstocks, we have assumed that plants were made of four types. The simulation starts with the instantiation of a stem (type 1) that will elongate and form adventitious roots (type 2) during four days (based on our experimental observations). At day 4, the stem is transformed into a new type of stem (type 4) that does not produce any adventitious roots. The adventitious roots start elongating after a developmental lag phase and form lateral roots (type 3)

throughout their life following an acropetal sequence. Senescence was not simulated because it was not observed in our phenotyping experiments.

6.3. Results and discussion

6.3.1. Plant phenotypes

The plant population was used in previous studies to analyze the rootstock-mediated variation in tomato vegetative growth under potassium, phosphorous, drought, salinity and soil impedance stresses (Albacete *et al.*, 2015a; Albacete *et al.*, 2015b) and in other genetic studies (Asins and Carbonell, 2014; Asins *et al.*, 2015; Kevei *et al.*, 2015). The root phenotyping experiment has generated a collection of root system images for each line of the grafted population, from which a number of morphological descriptors have been computed (see Chapter 5). Some of these descriptors provided a direct estimate of a root variable (*e.g.*, longest root length, root system width), while others were derived from image analysis (*e.g.*, foreground pixel density in defined areas). In total, ca. 2700 plants were analyzed and an important morphological diversity was observed within the grafted populations (**Figure 6-1**). The observed diversity appeared to involve all developmental and growth processes underlying root system architecture.

In the present approach, we aimed at obtaining descriptors of the dynamics of the root system, using the RootTyp model (Pagès *et al.*, 2004). An illustration of a manual fitting of RootTyp to two contrasting lines was show in **Figure 6-2**.

Chpter 6



Figure 6-1. Illustration of the morphological diversity observed in aeroponics within the tomato population (top left: narrow branching angle of adventitious roots; top center: large branching angle of adventitious roots; top right: plagiotropic lateral roots; bottom left: lack of gravitropism (exotropism); bottom center: long lateral roots; bottom right: large number of adventitious roots).



Figure 6-2. Example of two root system images obtained at the end of the experiment from two contrasting lines differing by the rate of production of adventitious roots (left and right panels). The center panel illustrates root systems of the same lines simulated using the model RootTyp after manual fitting of the parameters.

123

6.3.2. Plant modeling workflow

As aeroponics provides a uniform root environment, the coefficients fixing the influence of the soil on growth and branching were set at a value of 1.0 (*i.e.* no soil influence), the soil impedance was set at 0.0 and soil anisotropy was disabled. Soil depth was set at -300 cm.

In a first step, we created a generic tomato rootstock growing after excision of the root system and based on the four types described above. For each type, the parameters which did not vary in the population, or which can not be estimated from the available data, were set empirically at a fixed value (*e.g.* root diameter, duration of lag phase). The remaining parameters, which we will try to estimate in this study, comprise the distance between successive adventitious roots along the stem segment (hence the number of adventitious roots), the initial growth rate of the adventitious roots, the insertion angle and tropism strength of the adventitious roots, the growth rate of laterals and their insertion angle and tropism (**Table 6-1**).

Estimation of stem parameters

The inter-branch distance and growth rate of the stem (type 1) have been computed in a way that the stem would produce the observed number of adventitious roots (type2) during the first four days. The diameter is set as a constant value of 0.518 cm. Initial growth was placed arbitrarily to 1.5 cm.j⁻¹ with a null standard deviation so that the stem is 6.0-cm long when the transition to type 4 occurs. The average maximum length is set at 999.0 cm and a standard deviation of 0.0 so that the stem the growth rate is constant during the first 4 days.
Chpter 6

Characteristic	Unit	Stem	Adventitious	Lateral	Transition stem
Туре					
Type Number	N.A	1	2	3	4
Growth					
Average insertion angle	Rad	3,141	1,57	1,57	3,141
Standard deviation	Rad	0	0	0	0
Primordia development period	Day	0	4	4	0
Initial average growth	Cm/Day	1,5	G.D	0,5	0
Standard deviation	Cm/Day	0	G.D	0	0
Maximum root length	Cm	999	G.D	G.D	999
Standard deviation	Cm	0	G.D	G.D	0
Senescence period	Day	17	17	17	17
Diameter	Cm	0,518	0,107	0,029	0,518
Branching					
Inter-branching	Cm	G.D	G.D	N.A	N.A
Standard deviation	Cm	G.D	G.D	N.A	N.A
Subtype name	N.A	Adv.	Lat.	N.A	N.A
Occurrence probability	N.A	1	1	N.A	N.A
Growth direction					
Tropism type	N.A	-1	1	1	-1
Tropism strength	N.A	2	G.D	0,5	2
Sensitivity to mechanical constraint	N.A	0	0	0	0
Transition phase					
Age	Day	4	N.A	N.A	N.A
Occurrence probability	N.A	1	N.A	N.A	N.A
Transition name	ΝΔ	тѕ	ΝΔ	ΝΔ	ΝΔ

Table 6-1. Root parameters of the stem, adventitious root, lateral root and transition stem for growth, branching, growth direction and transition phase in RootTyp software.

N.A: Not Applicable; D.G: Genotype Dependent – estimated by inverse modelling; Adv: Adventitious; T.S: Transition stem; Lat: Lateral

Estimation of adventitious root parameters

The insertion angle was not measured directly on the images. Instead it was evaluated manually along a discrete scale from 1 (parallel to the parent axis) to 5 (orthogonal to the parent axis), with an amplitude of 18 ° for each category. The category was converted to an insertion angle in radians with the following formula:

Insertion angle = $(1 - adventitious root angle/5) * (\pi/2)$

Since most of the adventitious roots emerged after 4 days, the emergence duration was set to 4 days. The diameter was also considered as constant and fixed to the measured average value of 1.07 mm.

The initial root growth rate parameter was dividing the maximum length by the duration of the experiment. In doing so, we assumed that (i) the longest root is the first formed on the stem, (ii) the expected value of growth rate is equal for all adventitious roots and (iii) the growth rate is stable over time.

The interval between lateral roots formed along adventitious roots has been computed as follows. During the image analysis, the interval between the lateral roots was characterized by scores ranging from 1 (0 to 150 pixels) to 6 (751 to 900 pixels). The interlateral distance was computed as the mid of the interval corresponding to the score:

Inter Lateral Distance = 0.27 + (class interval - 1) * 0.54

The type of tropism was set to 1 because all adventitious roots had ultimately a vertical growth (due to a combination of positive gravitropism and natural bending due to their hanging in the air).

The tropism parameter of RootTyp is a non-dimensional number which used to size a tropism vector that is added to the growth direction to change the direction. Since the effect of tropism on angle depends on the growth rate, a number of simulations were made with the generic tomato plant in RootTyp to calibrate a linear relationship between the three variables The R² of the regression was equal to 0.77. A first set of simulations were carried out with elongation rates of 0.1; 1; 2; 3; 4; 5; 6; 8 and 10 cm.d⁻¹ and tropism strengths of 0 to 4 (by steps of 0.5). The second series of simulations was performed by keeping a constant growth rate 5 cm.d⁻¹ and varying only the tropism with values of 0 to 4 (by steps of 0.1). Each simulation was set to produce 6 adventitious roots. The width of the root system was measured on each simulations, reproducing the protocol of Chapter 5. A linear regression was performed in order to predict tropism on the basis of root growth and root system width. The best R² (0.77) was obtained with the following equation where all parameters were significant (P<0.001):

$$Tropism = 11.2 - (1.24 * log(Width)) - (2.61/Initial growth)$$

Estimation of lateral root parameters

As mentioned earlier, lateral roots emerged orthogonal to their parent root and we did not observe any differences between different genotypes. Accordingly, the angle of insertion has been set at 1.57 radians. The duration of development of lateral root primordia was set at 4 days. We did not make specific observation of this parameter and used a value generally measured on several species. The diameter was assumed to be independent of genotype and was fixed at the measured diameter of 0.29 millimeter. As said earlier, the lateral roots did not produce roots. The tropism has been set to 1 as for adventitious roots, however, the weight of tropism was placed arbitrarily 0.5 to mimic the slight curvature observed on most of the pictures.

The information available to evaluate the growth of lateral roots is the temporal evolution of the root density recorded in the pictures, evaluated as the proportion of white pixels in a region of interest of the image (see chapter 5). In order to generate an initial growth rate and maximum length for lateral roots, and due to the lack of detailed information on early root growth rate, we assumed that the initial growth was equal to 0.5 cm.d⁻¹ for all genotypes, so that only the maximum length of lateral roots had to be estimated.

In order to extract lateral root length from root density data, we had to consider the other factors which influence root density, viz. the number of adventitious roots and the interval between lateral roots. As for the direction of growth of adventitious roots, a series of simulations were performed to establish an empirical relationship between the number of white pixels and the maximum length of lateral roots. These simulations used the following parameters: (i) the length of lateral roots was varied from 0 cm to 15 cm, (ii) the number of lateral roots was varied 5 to 40 and (iii) the distance between laterals was varied from 0.27 to 2.97. The growth of adventitious roots was determined with an initial growth of 2.5 cm.d⁻¹ and a tropism strength 1. For each simulation, the root density was evaluated by reproducing the protocol used in Chapter 5 as the proportion of white pixels in a corresponding region of interest. For given number of adventitious roots and interval between laterals, the function that best described the relationship between the number of pixels and the length of lateral roots was a logarithmic regression with two parameters (slope and intercept). These two parameters were estimated for each of all combinations of number of adventitious roots x interval between laterals. Afterwards, we could estimate a relationship between the slope/intercept with the number of adventitious roots and the interval between the roots. The following set of empirical equations were finally used to determine the length of lateral roots, with an R² of 0.80:

The maximum length of lateral roots = $e^{(Intercept+(Slope*Root density))}$ Intercept = -0.454 * Log(LR Interval) - 0.051 * Adv. Root number Slope = 18.213 + 3.088 * Log(LR Interval) - Log(Adv. Root number)

6.3.3. Results of the parameterization of the whole rootstock population

The parameterization has been made for all lines of the grafted population under low and high N levels. For 26 genotypes in low N and 22 genotypes in high N, at least one of the parameters could not be obtained (typically because the image configuration did not allow the observation of some information). Virtual root systems generated by RootTyp using these parameters are illustrated on **Figure 6-3** for contrasting lines.

To assess the quality of the root curvature modeling, the width of the simulated root architecture was compared with the average value for each genotypes. The model overestimated each time the observed width of the root system. This character is based on three parameters: the tropism strength, the initial growth rate and the maximum length. We verified that the simulations used to calibrate the regression were in the same range as the experimental data with which this relationship was used. Width values ranged from 124 to 13.148 pixels in the simulations and from 250 to 3500 pixels in the experiment, and growth values ranged from 0.1 to 10 cm.d⁻¹ in the simulations, with real values between 1.0809 and 9.1324 cm.d⁻¹ in the experiment. The domain of simulation was thus appropriate. Another possibility to explain the overestimation is the fact that the maximum length of adventitious root was set at 999 cm during the calibration of the regression, and at the actual maximum root length during the estimation of genotype parameters. This might have led to a smaller estimate of the growth rate for the calibration simulations, and thus a stronger tropism.





We have also computed the root volume for virtual root systems of each genotype and have related this predicted volume with the observed fresh root mass. There is a relatively low correlation between the simulated volume and weight (with R² of 0.37 fresh weight). However, a few extreme values were found to drastically reduce

Chpter 6

the quality of the relationship and a new regression was performed excluding the outliers, identified using Cook's distance. Fifteen submissions were rejected. In the remaining dataset, the correlation accounted for 50% of the variability, as illustrated on **Figure 6-4**. The quality of the relationship between the actual and simulated values remain low because of numerous assumptions and approximations raised during the modeling and the poor quality of the image analysis routines. Given these shortcomings, the results look rather promising.



Figure 6-4. Plot of the observed root fresh weight (g) as a function of the simulated root volume (cm^3), for each genotype and conditions ($R^2 = 0.50$).

6.4. Conclusion

We have developed a modelling pipeline to extract dynamic parameters of root system growth and development from a single image and we have tested this pipeline on a large collection of genotypes grown in contrasting environments. The pipeline seems to reproduce a gross trend across the population and N levels. However, the limited value of the validation suggests that this information should not be trusted to predict details of the root architecture of individual genotypes. We anticipate that the quality of the simulations would be dramatically improved with an improved chain of image capture and analysis, whose development is ongoing.

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Authors' contributions

G. Lequeue performed experiments, analyzed data and drafted the manuscript. B. De Leener helped to analyzed data. X. Draye helped to design the study and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflicts of interest. The views and opinions expressed in this article are purely those of the writers and may not in any circumstances be regarded as stating an official position of the European Commission.

Chapter 7

Conclusions and perspectives

The aims of this PhD was to investigate the genetic determinism of root system architecture (RSA) in grafted tomato (*Solanum lycopersicum* L.) under nitrogen deficiency, in relation with biomass production. To this end, two action lines were simultaneously developed. The first consisted in developing nitrogen monitoring methods (Chapter 3 and 4), while the second consisted in a genetic analysis of RSA and growth under low and high nitrogen, using a novel aeroponic root phenotyping platform and a custom model-based image analysis pipeline (Chapter 5 and 6).

We introduced a low-cost, high throughput method to measure plant nitrogen based on Near-infrared microscopy (NIRM) and demonstrated the feasibility of accurately estimating the N content of very small tomato leaf samples using NIRM. The main benefits of this technique compared to conventional methods (*e.g.* Kjeldahl, Dumas and NIRS) lays essentially in the ease of sample preparation, in the small amount of tissue that is required and in the reduced analytical costs and times. This new method should ease (i) the establishment of N profiling among different organs of the same plant, (ii) the dynamic monitoring of N content in time for a given plant and (iii) the development of high throughput methods of N quantification in studies involving large numbers of genotypes.

In parallel to the development of this novel method, we assessed optical methods to monitor nitrogen content evolution non-destructively in tomato. The reflectance sensing devices offered a good way to detect N variability between N treatments and

133

developmental stages. Induced fluorescence methods, appeared to outperform active reflectance methods. The first reason is that reflectance provides information on the chlorophyll content, whereas induced fluorescence returns two leaf pigments, phenolics and chlorophyll content whose concentrations are influenced by the N status of the plant (Mercure *et al.*, 2004; Cartelat *et al.*, 2005; Solari *et al.*, 2008; Longchamps and Khosla, 2014). The second reason results from the fact that chlorosis on upper leaves occurs very late in the nitrogen deficiency syndrom (Kitchen *et al.*, 2010; Longchamps and Khosla, 2014), while early changes in phenolics in upper leaves can be detected through higher UV absorption (Crankshaw and Langenheim, 1981; Samson *et al.*, 2000; Longchamps and Khosla, 2014).

In the genetic analysis developed, an important morphological variability was observed within the grafted population. This variability concerned all major developmental and growth processes underlying root system architecture. Interestingly, the heritability for the inter-lateral distance indicates that the genetic determinism of lateral root initiation is largely different at low and high nitrogen conditions. This is an important result as lateral roots are known to respond to N supply and plant N status and contribute to much of the absorptive surface of the root system.

It is widely admitted that biomass production decreases under nitrogen stress. In this study, four genotypic groups could be assembled based on their shoot biomass. Breeders are likely interested in genotypes that have high biomass regardless of the treatment, or that have an average biomass in normal conditions and a biomass above average under stress. On the root side, we observed globally an increase in root length and a decrease of fresh weight, leading to an increase of the specific length, as in the experiments of Miller and Cramer (2005). Most probably, this implies a redistribution of the root volume from the top profile towards faster root growth at depth.

Chapter 7

A rather small number of QTLs, most with small LOD score values, were observed for the three major processes which shape root system architecture, viz. root growth rate, root branching and root tropism. Small LOD scores in aeroponics have been commonly observed in maize, barley, wheat and rice (in our laboratory). This suggests that, in the absence of mechanical, gas or nutrient constraints, which is typical of aeroponics, large collections of genotypes within a species tend to display a narrow range of phenotypes. In view of many very large root QTL effects reported in the literature in soil or other conditions, this would indicate that the scope for root architecture improvement lays more in the root responses to environmental constraints than on constitutive traits. If this was to be the case, the scientific community should probably reconsider much of the actual root architecture phenotyping practices.

Tomato breeding aims at delivering new cultivars, plants and rootstock, with improved agronomical traits. Novel tomato cultivars are specifically targeted to a particular set of growing conditions, such as soilless culture, high tropical temperatures, and field or greenhouse conditions. Yet breeding objectives evolve over time with the modifications of growing systems, of the incidence of environmental constraints such as pests and diseases and of consumer preferences in terms of quality (Kubota *et al*, 2008; Lee *et al*, 2010; Bergougnoux, 2014).

Understanding the mechanisms supporting an interesting agronomic trait has sometimes been useful for breeders. More recently, quantitative trait locus (QTL) analysis has opened new avenues such as marker assisted selection, especially valuable if the trait background is unknown or if the trait phenotype is hardly accessible (Lander and Botstein, 1989; Collard *et al*, 2005), which is typically the case for many root traits. The main current challenge in horticulture is to improve plant productivity and product quality, with a special attention to environmental quality and human wellbeing (Masclaux-Daubresse *et al.*, 2010; Ben Abdallah *et al*, 2016). Such a goal depends on agricultural management and can be achieved by providing the right nutrient source at the right rate, the right time and the right place (Miao *et al.*, 2007; Masclaux-Daubresse *et al.*, 2010; Miao *et al.*, 2011; Diacono *et al.*, 2013; Ben Abdallah *et al*, 2016). To improve sustainable agricultural production, it is also necessary to grow crops that can remove the applied nutrient efficiently, and therefore require less fertilizer (Masclaux-Daubresse *et al.*, 2016). Such global « resource use efficiency » necessitates having a global view of the crop physiology, the plant uptake capacity, the plant metabolism and the plant response to constraints, as well as a view of soil physical and chemical properties (Masclaux-Daubresse *et al.*, 2013; Ben Abdallah *et al.*, 2016).

In the case of N deficiency, plants improve their ability to acquire these mineral elements by altering their carbon partitioning to favour root growth and by optimizing root morphology. Interestingly, one of the early physiological effects of N deficiency is a rerouting of primary metabolism and the accumulation of sugars in leaves (Hermans *et al.* 2006; Wang and Ruan, 2016). This rerouting increases the transport of sugars to the root, which serves to increase the R:S biomass ratio and, in tandem with changes in hormone concentrations, modifies root morphology (Hermans *et al.* 2006; Wang and Ruan, 2016). This enables plants to respond appropriately to N deficiencies and to forage more effectively for minerals with low availability in the rhizosphere (Druege *et al.*, 2004; Hermans *et al.* 2006; Zerche and Druege, 2009; Wang and Ruan, 2016). Furthermore, after storage under low temperature and light as in our experience, high nitrogen supply and high light conditions lead to an increase of endogenous nitrogen content, which has a positive

effect on rooting of the future cuttings (Druege *et al.*, 2004). However, this response is conditioned by carbohydrate availability as high nitrogen has no effect or even inhibits adventitious root formation when endogenous sugar content is low (Druege *et al.*, 2004; Zerche and Druege, 2009).

This research shows the much more complex link between different metabolic and physiological clues. The enzymes and regulatory processes that can be manipulated to control nitrogen use efficiency should be studied to allow their manipulations. The last results obtained from natural variation and QTL studies in this research and more widely in the RootOPower project, show the complexity of nitrogen use efficiencys. With regard to the complexity of the challenge we have to face and with regard to the numerous approaches available in research, the integration of data coming from high-throughput profiling of transcriptomic studies, functional genomics, quantitative genetics, ecophysiology and soil science into explanatory models of whole-plant behaviour in the environment has to be continued and encouraged. These approaches will be steps towards the creation of varieties that have improved mineral acquisition and make more efficient use of minerals, and the development of novel strategies for sustainable agriculture.

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139

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179

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Bompy F., Dulormne M., Dufay B., Mira E., **Lequeue G.**, de Girval N., Virapin V., & Imbert D. Seasonality effect on physiology and Growth performances of Caribbean mangrove tree species. *The 50th Anniversary Meeting Association for Tropical Biology and Conservation (ATBC)*, Organization for Tropical Studies (OTS), San José, Costa Rica, June 25, 2013.

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Communication at national conferences

Lambrechts T., **Lequeue G.**, Lobet G., & Lutts S. Impact of cadmium and zinc on root system of Lolium perenne and Trifolium repens. *The 18th national symposium on applied biological sciences*, Faculty of bioscience engineering, Ghent University, Gent, Belgium, February 08, 2013.

Posters presented at international conferences

Lequeue G., Draye X., & Baeten V. Determination by near infrared microscopy of the nitrogen content of tomato (*Solanum Lycopersicum* L.) leaf powder. *ICNIRS 2017: 18thInternational Conference on Near Infrared Spectroscopy* - NIR Spectroscopy at work in industry, University of Copenhagen, Copenhagen, Denmark, June 11 to 15, 2017.

Lequeue G., Baeten V., & Draye X. Determination by near infrared microscopy (NIRM) of nitrogen content in tomato (*Solanum lycopersicum* L.) leaf powder. *SOL 2015: 12th Solanaceae Conference*, INRA Bordeaux-Aquitaine and University of Bordeaux, Bordeaux, France, October 25 to 29, 2015.

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Curriculum vitae

Gauthier Lequeue was born on November 13th, 1985 in Tournai, Belguim. He enrolled at the University of Namur (Unamur), where he obtained a bachelor's degree in Biology in 2009, followed by a master's degree in Biology of organisms and ecology in a joint program with the University Catholic of Louvain (UCL) in 2011. During his master, he also did an internship of four months at the UMR EcoFoG-Dynecar at the University of Antilles and Guyana (Guadeloupe) in collaboration with Prof. Maguy Dulormne and Dr. Félix Bompy.

After that, he worked for one month in fertilizer manufacturer and for three months in a familial nursery specialized in the production of Christmas tree.

In May 2012, he started on his PhD in the RootOPower EU project in collaboration with Prof. Xavier Draye (UCL). From this PhD project, he was first author (ORCID: 0000-0001-8358-8134) of one peer-reviewed publication as well as co-author of two peer-reviewed publications and one national publication. He also participated in three international congresses and three national congresses.

183